REMARKS

Claims 1-16 are pending in the subject application. Applicant confirms the election with traverse of group I claims 1-16 in response to the restriction requirement and cancel claims 17-36 without prejudice or disclaimer. Applicant confirms the election with traverse of naproxen as a species selection. The amendment to claim 12 is made merely to correct a typographical error; no new matter is added to the claims.

1. THE CLAIMS AS AMENDED ARE NOT-OBVIOUS UNDER 35 U.S.C. § 103(A).

While disagreeing with the Examiner's analysis with respect to each of the obviousness rejections on the record (specifically set out below), Applicants have amended the claims as suggested by the Examiner to focus on a specific subset of hop acid moieties (*see for example newly amended Claim 1 now reciting* reduced isoalpha acids, dihydro-isolalpha acids, tetra-hydroisoalpha acids, and hexa-hydroisoalpha acids) in the interest of advancing the case to allowance. Reconsideration of all the claims in light of the proposed amendments and the following remarks are respectfully requested.

Claims 1-16 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 99/44623 ("WO") (abstract; page 4, lines 3-15; page 6, lines 6-20). Applicants respectfully traverse the ground of these rejections.

WO purports to teach,

> *"preparations and extracts of valerian, as well as isovaleramide, isovaleric acid, and its pharmaceutically acceptable salts, esters, and substituted amides, and other valerian-related compounds, in combination with NSAIDs [which are said to] exhibit clinically significant pharmacological properties which implicate a treatment for acute muscular aches, strains, and sprains which occur from a localized, external insult to a particular muscle [...]."*

Newly amended Claims 1-16 are limited to reduced isoalpha acids, including dihydro-isoalpha acids, tetra-hydroisoalpha acids, and hexa-hydroisoalpha acids. WO does not teach or suggest reduced isoalpha acids, dihydro-isoalpha acids, tetra-hydroisoalpha acids, and hexa-hydroisoalpha acids or methods of using the same. It is noted that none of the compositions and methods set forth in the WO reference could possibly include a reduced isoalpha acid preparation

alone or in combination with any of the many compounds named in that case. Reduced isoalpha acids are produced either through the hydrogenation of isohumulone, an iso-alpha acid, using palladium on carbon as a catalyst or through a direct isomerization of tetrahydrohumulone. Isohumulone, used for the hydrogenation process to form tetrahydroisohumulone, is generated by the boiling of hops with wort at a pH value of about 5.5. Under these conditions the alpha acids are transformed to the iso-alpha acids which may then be used in the hydrogenation process to form tetrahydroisohumulone, for example. Thus, the production of tetrahydroisohumulone via hydrogenation of isohumulone is two processing steps removed from the alpha acids of a hops extract. Additionally note that the direct isomerization route of production requires tetrahydrohumulone isolated from hops followed by the isomerization process. Again, as with the hydrogenation process, this route of production is at least two process steps removed from the presences of alpha acids in an extract (see, Chemistry and Analysis of Hop and Beer Bitter Acids, Elsevier 1991, Eds: Verzele, M., and De Keukeleire, D. Chapters 5 and 6, provided herewith as Attachment A).

In addition, the Examiner's attention is directed to the supragenus structure of claim 3 and Genus A & B structures of claims 4 and 5 respectively. Examination of the structures indicates that isovaleramide (as per WO) cannot be formed insofar as the named structures lack a nitrogen atom requisite for the amine formation. Additionally note that producing isovaleric acid from the described generic structures would require cleavage from the furanose ring at the carbon atom of the double bond site intermediate to the two double bonded oxygen followed by a subsequent hydroxylation at that cleavage site. The Applicants respectfully submit that such cannot occur via the routes of oxidation and enzymatic action as attributed to WO at page 11 lines 17 *et seq.*

Claims 1-16 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over JP 406312924 ("JP '924") or JP 04202138 ("JP '138") taken with Sunshine *et al.* (U.S. Patent No. 4,780,463 (the "'463 Patent") or CA 2175091 ("CA '091"). Applicants respectfully traverse the ground of these rejections.

JP '924 and JP '138 at best purport to teach the use of hops. JP '138 is said to teach a method for the extraction of lupuronic acid, coluprone and adluprone. Each of these are beta acid preparations which could not include reduced isoalpha acids according to the invention set forth in

the claims as amended. The JP '924 Abstract purportedly relates to cohumulone, humulone, and adhumulone preparations obtained by the Wollmer's method. The JP '924 Abstract does **not** teach or suggest reduced isoalpha acids. In fact, the formulations of the JP '924 Abstract cannot include reduced isoalpha acids since this compound is not naturally found in hops but is obtained by modifying hops acids (e.g., by reducing existing compounds). The abstract does not teach or even suggest modifying the chemical moieties relied upon as antioxidant. Kindly note that the Wollmer's method (which relies on lead acetate) could not possibly produce reduced isoalpha acids. Also, this Abstract does not teach or suggest combining modified hops acids with NSAIDs according the claims as amended.

The CA '091 Abstract relates to a controlled release tablet of naproxen (an NSAID) as an anti-inflammatory modality and does not teach or suggest a combination with a reduced isoalpha acid according to the claims as amended.

The '463 Patent relates to naproxen combinations with other known analgesics. However, the '463 Patent does not teach combinations with hops and more specifically with reduced isoalpha acids.

Applicants thus, assert that (a) none of the cited references teaches or suggests the combination of an NSAID with a reduced isoalpha acid; and that (b) no combination of the references in any possible permutation teach or suggest compositions or methods relying on the combination of an NSAID with a reduced isoalpha acid. Applicants therefore do not reach the issue as to whether there was a motivation to combine since the combination in itself fails to arrive at the invention of the claims as amended. It is also noted, that there is no motivation to further modify the teachings of any of the cited art to arrive to reduced isoalpha acids. For these reasons, Applicants submit that the instant invention is not obvious over the art cited and respectfully request withdrawal of these rejections.

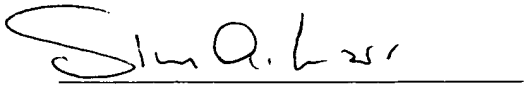
CONCLUSION

It is submitted that the amended claims are patentable over the teachings of the prior art relied upon by the Examiner. Accordingly, favorable reconsideration of the claims is requested in light of the preceding amendments and remarks. Allowance of the claims is courteously solicited.

If there are any outstanding issues that might be resolved by an interview or an Examiner's amendment, the Examiner is requested to call Applicant's attorney at the telephone number shown below.

Pursuant to 37 C.F.R. § 1.136(a)(2), the Examiner is authorized to charge any fee under 37 C.F.R. § 1.17 applicable in this instant, as well as in future communications, to Deposit Account 50-1133. Furthermore, such authorization should be treated in any concurrent or future reply requiring a petition for an extension of time under Section 1.136 for its timely submission, as constructively incorporating a petition for extension of time for the appropriate length of time pursuant 37 C.F.R. § 1.136(a)(3) regardless of whether a separate petition is included.

Respectfully submitted,
McDERMOTT WILL & EMERY LLP



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Date: November 20, 2006
(November 19, 2006 fell on a Sunday)

CHAPTER 5
THE ISOHUMULONES

In the brewery, hop is boiled with wort at a pH value around 5.5. In these conditions the hop alpha acids are poorly soluble (1), but in the process they are transformed into the iso-alpha acids, which are better soluble in the wort medium (2). Consequently, only traces of alpha acids remain in beer (3) (see 5.3.). The iso-alpha acids are the hop derivatives, which contribute mainly to the beer bitter taste. In this Chapter the chemistry of the isohumulones, which are the most important iso-alpha acids, is discussed. The chemistry of the other iso-alpha acids is practically identical to that of the isohumulones. As humulone is readily available, it is easier to study the isohumulones than the other iso-alpha acids. Therefore, the isohumulones are the best known iso-alpha acids.

5.1. STRUCTURE OF THE ISOHUMULONES.

5.1.1. ISOLATION.

More than 50 years ago Wieland described the hydrolysis of humulone to humulinic acid, isohexenoic acid and isobutyraldehyde (4). He proposed an intermediate, which was later on recognized as isohumulone. Soon thereafter Windisch, Kolbach and Schleicher (5) obtained a resinous oil upon boiling humulone in buffers or in dilute alkaline solutions. They agreed with the structure proposed by Wieland and coined the name "Soft Resin A". These investigators also suggested that hydrolysis yielded either humulinic acid and isohexenoic acid or isobutyraldehyde and a "Resin B", which afterwards turned out to be acetylhumulinic acid (see 8.3.). This substance would immediately be converted to humulinic acid and acetic acid in the alkaline conditions. About twenty years later this work was resumed by Verzele and Govaert, who isolated two crystalline products upon hydrogenation of isomerized humulone and from hydrogenated beer extract (6). They proposed that "Resin A" should be called isohumulone, a name which has since been generally adopted.

Investigation of the beer bitter components with counter-current distribution (CCD) revealed a mixture of mainly three analogues, corresponding to the hop alpha acids (7). The distribution pattern of isohumulone, obtained by alkaline isomerization of humulone, showed that the bands were about 15% broader than the calculated curves (8). Using reversed phase partition chromatography, Spetsig was able to separate the components, obtained upon boiling of humulone in a buffer solution with pH 5.0, into

two components (9). The bitter substances of beer gave six peaks, i.e. two for each important hop alpha acid. Such analytical separations have also been carried out by paper chromatography (10,11) and more recently with HPLC, as detailed in the Chapters 15-17.

Preparative isolation of the two isohumulones was achieved by reversed phase partition chromatography (chloroform as stationary phase on Hyflo Super Cel; buffers with 25% methanol as mobile phase) (12) and by CCD (3000 transfers in the two-phase system iso-octane : aqueous buffer pH 5.5) (13). Originally, the compound in the band with K 0.61 was called isohumulone A, the compound of the band with K 0.764 isohumulone B. This was unfortunate, considering the possible confusion with 'Welchharz A' and 'Welchharz B' of Windisch (5). Afterwards, the compounds were appropriately named trans and cis isohumulone (see 5.1.2.). The isohumulones have also been separated on a preparative scale by thin layer chromatography (Kieselgel H; benzene : ether 16:1 as eluent) (14).

The capacity of the just cited chromatography procedures was very limited, mostly in the sub-milligramme range. CCD, however, can achieve the separations on gram amounts (15). This was essential for the success of much of the work of our laboratory.

Larger scale preparative chromatography of the isohumulones and other iso-alpha acids has been mentioned recently (16) and is described in detail in Chapters 15-17.

5.1.2. NOMENCLATURE.

For the sake of uniformity in nomenclature, rules have been worked out by the Hops Liaison Committee in cooperation with the European Brewery Convention and the American Society of Brewing Chemists (17). Too often these rules are disregarded in the literature, where for example the term 'isohumulones' is loosely used to indicate the 'iso-alpha acids'. In a recent example of this (18), the term n.-humulone is furthermore used to designate humulone. This is required of course if the term 'humulones' is used instead of the name 'alpha acids'. It is most frustrating to be confronted with terms like 'the magnesium humulates' and 'the trans-isohumulones' when the magnesium salts of the alpha acids and the trans iso-alpha acids are the terms to be used. Editors of Journals should be more strict in demanding that the rules are followed and should refuse papers which do not conform with the officially accepted nomenclature (17). 'Isohumulones' are the mixture of the cis and trans isohumulones and do not include the co- and ad-derivatives. 'Humulone' is one of the alpha acids and 'humulones' in the plural form is therefore a nonsense term.

For most of the five-membered ring hop compounds, such as the isohumulones,

two epimers (cis-trans isomers) exist due to the presence of two chiral centres. The cis form is the one with the 3-methyl-2-butenyl side chain (at C-5) and the tertiary hydroxyl group (at C-4) on the ring skeleton, located on the same side of the plane of the ring. In the trans form these reference groups are oriented to opposite sides of the ring. Thus cis isohumulone (previously isohumulone B) with the 3-methyl-2-butenyl side chain and the 4-methyl-3-pentenoyl side chain in trans position (65, Fig. 36) is the thermodynamically most stable compound as the steric hindrance between the large groups is smallest.

5.1.3. STRUCTURAL PROOFS.

The structural formula of isohumulone had already been proposed by Wieland (4) and was confirmed, with only a minor change, much later (19). The exact stereochemistry was determined in 1971 (20).

5.1.3.1. THE RELATIVE CONFIGURATION.

The ^{1}H NMR spectra of both isomers display the appropriate signals for the protons in the 3-methylbutanoyl group, one 3-methyl-2-butenyl side chain and one 4-methyl-3-pentenoyl entity. Distinction can be made between the epimeric isohumulones. The least hindered, more stable cis compound clearly shows a more simple pattern (21). The geminal dimethyl groups of the alkenyl side chain at C-5 occur as a singlet in the cis form and as two singlets in the trans form. The ring proton at C-5 is a distinct triplet for cis isohumulone and a doublet of doublets for trans isohumulone. Also, the methylene protons of the alkenyl side chain at C-5 absorb at different δ values in trans isohumulone, forming an AB-spin system. In cis isohumulone a simple triplet signal is observed.

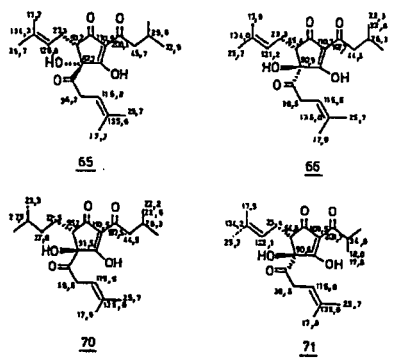
Cis isohumulone.

^{1}H NMR (100 MHz; $CDCl_3$; TMS) : δ : 0.97 (6H, d, J = 6 Hz); 1.58 (6H, s); 1.64 (3H, s); 1.72 (3H, s); 2.2 (1H, m); 2.43 (2H, t, J = 6.5 Hz); 2.73 (2H, d); 3.19 (1H, t, J = 6.5 Hz); 3.31 (2H, d, J = 6.5 Hz); 5.01 (1H, t, J = 6.5 Hz); 5.19 (1H, t, J = 6.5 Hz); 8.7 (2H, s).

Trans isohumulone.

^{1}H NMR (100 MHz; $CDCl_3$; TMS) : δ : 0.94 (3H, d, J = 6 Hz); 0.97 (3H, d, J = 6 Hz); 1.54 (6H, s); 1.68 (3H, s); 1.72 (3H, s); 2.15 (1H, m); 2.40 (2H, m); 2.70 (2H, d, J = 7 Hz); 3.02 (1H, 2 x d, J = 6 and 9 Hz); 3.30 (2H, d, J = 7 Hz); 5.12 (1H, t, J = 7 Hz); 5.18 (1H, t, J = 7 Hz); 8.6 (2H, s).

The ^{13}C NMR shifts of the cis and trans isohumulones are shown in Fig. 36. An unequivocal assignment of the carbonyl resonances is not possible, except for the 3-methylbutanoyl side chain. The chiral carbon atoms are shifted to higher field (3 to 6 δ units) in the cis isomer. Since this phenomenon is observed also in the ^{13}C NMR spectra of the humulinic acids (see 8.4.1.2.), it can be applied as stereochemical criterion. An analogy can be made with the 3-methyl-2-norbornanols provided the 3-methyl-2-butenyl side chain behaves as a methyl group and the conformation is to a first approximation planar. The C-2 and the C-3 carbon shifts are calculated from the respective δ values of the 2-methylnorbornanes and the 2-norbornanols, based on simple additivity (22). Differences between the calculated and the experimental values should reflect the mutual interaction of the substituents. The observed shifts for the C-3 carbon atom are indeed about 6 δ values lower in the cis derivatives and about 0.25 δ in the trans compounds. These results agree with the data for cis and trans isohumulones. Eclipsing of vicinal alkyl and hydroxyl groups causes an extra shielding of the carbon atoms carrying these substituents.



65 66 70 71

Fig. 36. Structural formulae and ^{13}C NMR data of the isohumulones (65,66), trans dihydro-isohumulone (70) and trans isocohumulone (71).

Individual assignments of the carbon atoms in the three different side chains follow from comparison with trans dihydro-isohumulone (70, Fig. 30) and trans isocohumulone (71, Fig. 36). The results are controlled for trans isohumulone by means of the coherent off-resonance decoupling technique with variable offset (Table 7; see 2.1.1.2.1.). The correlation between the ^{13}C NMR signals and the ^{1}H NMR signals is excellent.

The UV absorption maxima of the isohumulones are situated at 227 (ε 10200) nm and 279 (ε 11150) nm in acidic methanol and at 251 (ε 18300) nm with a shoulder at 270 (ε 14900) nm in alkaline methanol.

The component with the smallest distribution coefficient in a two-phase system, consisting of a hydrocarbon and an aqueous buffer, after recrystallization from iso-octane, has a melting point of 65°C and a specific rotation at the Na_D line of -7.8 in methanol. This compound is trans isohumulone or trans 2-(3-methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(4-methyl-3-pentenoyl)-2-cyclopentenone (66, Fig. 36).

Table 7. Relation between the experimental ^{13}C NMR resonance frequencies and the calculated ^{1}H NMR resonance frequencies.

$\delta\ ^{13}C$	$\delta\ ^{1}H$ (calculated)
17.9	1.25
22.6	0.82
23.3	2.33
25.7	1.65
26.3	2.12
38.5	3.30
44.5	2.68
55.4	2.95
115.5	5.15
121.2	5.15

The isomer with the highest distribution coefficient occurs usually as a light-yellow oil. By avoiding acidification of the aqueous phase upon isolation of the relevant CCD band, cis isohumulone or cis 2-(3-methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(4-methyl-3-pentenoyl)-2-cyclopentenone (65, Fig. 36) can be obtained in

crystalline form (23). It has, after recrystallization from iso-octane and filtration in the cold, a melting point of 18°C (20) and a specific rotation at the Na_D line of +47.6 in methanol.

5.1.3.2. THE ABSOLUTE CONFIGURATION.

The absolute configuration of the isohumulones has been established by chemical modification, combined with an empirical method to determine the absolute configuration (20), and by means of a study of the circular dichroism spectra (24). Previously, the absolute configuration was erroneously derived without further proof (25).

a) By chemical modification (19).

(i) Formation of the dihydrodeoxohumulinic acids and the corresponding methyl enol ethers.

To obtain simple, well-characterized derivatives, the epimeric isohumulones have been transformed into the epimeric humulinic acids (see 8.4.) and further into the epimeric dihydrodeoxohumulinic acids (cis form : 72, Fig. 37; trans form : 73, Fig. 37). This last transformation is achieved by hydrogenolysis of the humulinic acids (26). The yield can be increased to 80-85% by carrying out the reaction in a 20%-solution of the humulinic acids in acetic acid with 5% Adams' catalyst at 55°C during 6-8 h. The optical activity is fully retained. The epimeric compounds 72 and 73 are separated by CCD in the two-phase system ether : aqueous buffer pH 7.65 after 100 transfers. The cis isomer 72 has a K-value of 1.36 and a melting point of 179°C. The UV absorption maxima are : 232 (ε 11800) nm and 271 (ε 18200) nm in acidic methanol, 255 (ε 18200) nm with a shoulder at 272 (ε 14900) nm in alkaline methanol. The separated compounds occur mainly as a mixture of keto-enol tautomers, which can be trapped as the corresponding methyl enol ethers upon treatment with diazomethane. Separation is achieved by gas chromatography or CCD in the two-phase system, consisting of iso-octane as upper phase and ethanol : water 3:2 as lower phase, after 2500 transfers. In both cases the compounds with the lowest K-value are present to the extent of 85%.

Structural confirmation follows from the spectral data. Distinction between the positional isomers is made by ^{1}H NMR spectrometry with the aid of the enone rule (27). Thus, the α-proton with respect to the keto-enol system is observed at highest field for the keto tautomer. Cis 2,5-bis(3-methylbutyl)-3-methoxy-4-hydroxy-2-cyclopentenone (74, Fig. 37) shows absorption of the proton at the chiral centre

carrying the secondary alcohol function at δ 4.81. The corresponding δ-value for the trans isomer (75, Fig. 37) is 4.12. These isomers are present in excess. The signal is at δ 4.38 for cis 2,4-bis(3-methylbutyl)-3-methoxy)-5-hydroxy-2-cyclopentenone (76, Fig. 37) and at δ 3.69 for the trans isomer (77, Fig. 37).

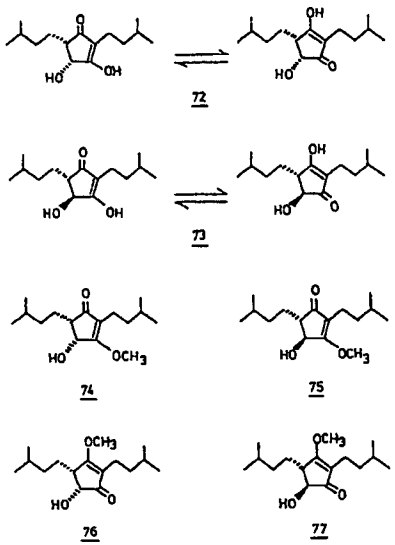


Fig. 36. Structural formulae of the dihydrodeoxohumulinic acids (72,73) and their methyl enol ethers (74-77).

Preparation and separation of the dihydrodeoxohumulinic acids and the corresponding methyl enol ethers.

Humulinic acid (2 g; 7.52 x 10^{-3} mol) is dissolved in acetic acid (10 g; 1.7 x 10^{-1} mol), to which 5% Adams' catalyst (platinum(IV) oxide) is added. The temperature of the hydrogenation vessel is adjusted to 55°C. After hydrogenation during 6-8 h the catalyst is filtered off and the solvent is removed. Trans dihydrodeoxohumulinic acid has a K value of 1.23 in the CCD two-phase system ether : aqueous buffer pH 7.65 after 100 transfers. The K value for the cis compound is 1.36. The epimers are treated separately with a slight excess of diazomethane. After 1h the solvent is removed and the residue is separated by GC or CCD in the two-phase system, consisting of iso-octane as upper phase and ethanol : water 3:2 as lower phase, after 2500 transfers. The K values of the predominating (85%) isomers 74 and 75 are 0.52. For the other isomers (15%) the K values are 0.62 for 76 and 0.64 for 77. The isolated compounds are light-yellow oils with absorption maxima around 245 nm.

Compound 74.

$[\alpha]_D$ (methanol, 20°C) : -26.3.

^{1}H NMR (60 MHz; CCl_4;TMS) : δ : 0.79 (6H, d); 0.80 (6H, d); 1-2 (9H, m); 2.01 (2H, t); 3.18 (1H, s); 4.08 (3H, s); 4.81 (1H, d, J = 7.0 Hz).
Mass spectrum (EI) : $M^{+\cdot}$ = 268.

Compound 75.

$[\alpha]_D$ (methanol, 20°C) : +11.53.

^{1}H NMR (60 MHz; CCl_4;TMS) : δ : 0.87 (12H, d); 1-2 (9H, m); 2.20 (2H, t); 2.9 (1H, s); 4.81 (3H, s); 4.38 (1H, d, J = 2.5 Hz).
Mass spectrum :(EI) : $M^{+\cdot}$ = 268.

Compound 76.

$[\alpha]_D$ (methanol, 20°C) : +29.5.

^{1}H NMR (60 MHz; CCl_4;TMS) : δ : 0.85 (6H, d); 0.87 (6H, d); 1-2 (9H, m); 2.11 (2H, t); 3.0 (1H, s); 3.98 (3H, s); 4.12 (1H, d, J = 7.0 Hz).
Mass spectrum :(EI) : $M^{+\cdot}$ = 268.

Compound 77.

$[\alpha]_D$ (methanol, 20°C) : +22.79.

^{1}H NMR (60 MHz; CCl_4;TMS) : δ : 0.88 (12 H, d); 1-2 (9H, m); 2.2 (2H, t); 2.6 (1H, s); 3.69 (1H, d, J = 2.5 Hz); 3.98 (3H, s).
Mass spectrum (EI) : $M^{+\cdot}$ = 268.

(ii) The relative configuration of the dihydrodeoxohumulinic acids.

One chiral centre in the epimers has necessarily the same absolute configuration. This can be identified by removing a chiral centre in both epimers and observing whether the resulting products display a similar ORD curve or a mirror image. The relative configuration was established by hydrogenolysis of the allylic alcohol function (see 8.4.3.1.2.4.) and by oxidation of this alcohol with bismuth(III) oxide (see 8.4.3.1.4.). It has been found in both cases that the reaction products are identical. The optical activity is retained because racemization would require the introduction of a second double bond or a second negative charge in the five-membered ring system.

(iii) The absolute configuration of the dihydrodeoxohumulinic acids.

The method of partial dedoubling of Horeau (28-35) was applied to establish the absolute configuration of the four enol ethers **74-77**. Since diastereomers, derived from reaction of a racemate with an optically active compound, are formed at a different rate, an excess of one enantiomer will be found in the remaining part of the original racemate. If the absolute configuration of the enantiomer in excess and, consequently, also that of the other enantiomer, which has reacted preferentially, are known, the absolute configuration of the optically active component can be derived. It is accepted that the least hindered diastereomer is formed preferentially.

In practice, racemic 2-phenylbutyric anhydride is used for the determination of the absolute configuration of chiral secondary alcohols. After esterification the excess anhydride is hydrolyzed and the optical rotation of the obtained 2-phenylbutyric acid is determined. The empirical rule is as follows : when (+) (S)2-phenylbutyric acid is isolated, the absolute configuration of the secondary alcohol is such that in the Fisher projection with the hydroxyl group left and the hydrogen atom right the sterically most hindered group is down. When (-) (R)2-phenylbutyric acid is isolated, the most hindered group in the same Fisher projection is up. It should be remarked that the sterically most hindered group does not necessarily correspond to the group with highest priority according to the Cahn-Ingold-Prelog nomenclature. The environment of the chiral centre must be sufficiently asymmetric in order to obtain a reasonable optical yield of 2-phenylbutyric acid. The Horeau-method, applied to compounds **74-77**, results in the ORD curves of the isolated 2-phenylbutyric acids, as displayed in Fig. 38.

Table 8. Chemical and optical yields of the esterification of compounds **74-77** with 2-phenylbutyric anhydride.

Compound	Chemical yield (%)	Optical yield (%)
74	68	5.1
75	100	1.1
76	64	11.7
77	100	9.9

The yields of the esterification reaction are lower and the optical yields are higher for the sterically least hindered cis compounds. The sterical encumbrance in **74** and **75** should originate in the difference between an alkyl group and a methoxy function. For **76** and **77** reference should be made to an alkyl group vs. a carbonyl function. Since the difference in the latter case is more pronounced, the optical yields are higher. It also follows that the optical yield is lowest for compound **75**.

These data are in agreement with the structural assignments and lead to the conclusion that the alkyl group is the sterically most hindered substituent. Since for the cis and trans derivatives 2-phenylbutyric acid with opposite absolute configuration is isolated, the chiral centre at the secondary alcohol function must have the opposite absolute configuration also. Application of the Horeau rule in the prescribed Fisher projection gives the (4R,5S)-configuration for **74** and **76** and the (4S,5S)-configuration for **75** and **77**.

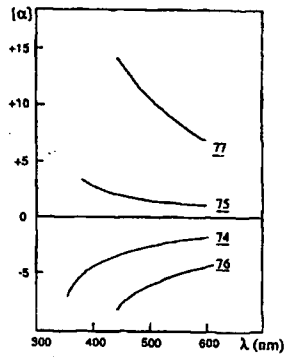


Fig. 38. The ORD curves of the 2-phenylbutyric acids, isolated from compounds **74-77** according to the Horeau method.

(iv) The absolute configuration of the isohumulones.

The absolute representations of the methyl enol ethers of the dihydrodeoxohumulinic acids can directly be transmitted to those of the humulinic acids and the isohumulones (see 8.4.1.3.). Cis isohumulone 65 has the (4R,5S)-configuration, trans isohumulone 66 the (4S,5S)-configuration.

The method of partial decoupling according to Horeau.

To the enol ether (10^{-4}) mol in pyridine (0.5 ml) is added 2-phenylbutyric anhydride (2 x 10^{-4} mol). After mixing and standing for 15-20 h at room temperature, a few drops of water are added, followed by heating on a steam bath during 30 min. The solution, after addition of water (2 ml) and benzene (3 ml), is titrated with NaOH 0.1 N on phenolphthalein. The aqueous layer is extracted (3 x) with benzene (10 ml) and subsequently acidified with HCl (2 ml). Then the optical rotation of the 2-phenylbutyric acid obtained is measured at 589 nm, 578 nm, 546 nm, 436 nm and 365 nm. The respective values are:

74 : -2.03; -2.15; -2.41; -4.5; -7.39.

75 : +0.62; +0.77; +0.93; +1.95; +3.59.

76 : -4.4; -4.7; -5.35; -9.0; (not measurable at 365 nm).

77 : +5.86; +6.11; +7.02; +12.4; (not measurable at 365 nm).

b) Study of the circular dichroism spectra (23).

Upon application of the Cahn-Ingold-Prelog notation (36) the carbonyl group of the acyl side chain at C-4 in the isohumulones has priority over the carbonyl group at C-3, because this carbonyl occurs almost exclusively in the enol form. This feature has been proved by study of the CD spectra. To evaluate the enolization pattern, the CD behaviour of compounds with fixed enolization has to be investigated first. The methyl enol ethers of the dihydrodeoxohumulinic acids (**74-77**) are suitable for this purpose.

Both the (n-π^*) or R bands and the (π-π^*) or K bands are useful to derive the chirality of cycloalkenones (37-40). A positive torsion angle around the non-planar transoid enone system in 2-cyclopentenones gives a negative Cotton effect for the R band (38,40,41). Since substituents on the enone chromophore may have a more substantial influence on the sign of the K band compared to the chirality of the system itself, only the more reliable R band has been interpreted. The CD spectra have been recorded in ethanol and iso-octane to detect changes due to solvent polarity. Fig. 39 shows the CD spectra for the cis components **74** and **76**, Fig. 40 displays the CD spectra of the trans compounds **75** and **77**.

The octant projections of the two non-planar conformations for the compounds **74** and **77** are given in Fig. 41. Conformations (a) lead to a positive Cotton effect, conformations (b) give a negative Cotton effect for the respective R bands. Since 1,3-diaxial interactions occur only between a σ-bond and a π-bond, an axial group is a priori not less stable than an equatorial position. The energy difference between the two conformers can indeed be very small (42). The *preferred* conformations are deduced from the observed signs of the Cotton effects.

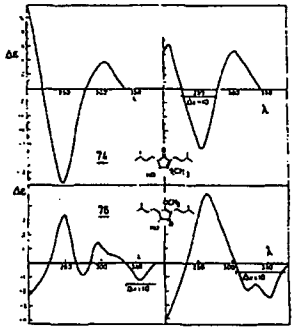


Fig. 39. CD spectra of the enol ethers of the cis dihydrodeoxohumulinic acids (**74,76**).

Compound **74** occurs in the conformation (a) with a pseudo-equatorial hydroxyl function and a pseudo-axial 3-methylbutyl group, while both substituents occur in the quasi-diequatorial conformation (b) for **75**.

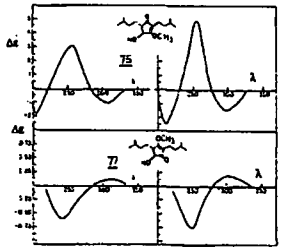


Fig. 40. CD spectra of the enol ethers of trans dihydrodeoxohumulinic acids (**75,77**).

The differences in magnitude of the $\Delta\varepsilon$ values are more pronounced in ethanol due to better solvation of the pseudo-equatorial hydroxyl groups. These features are however not relevant because the optical purity of the derivatives is unknown.

For **76** conformation (a) is preferred, i.e. with the hydroxyl function in pseudo-axial position and the 3-methylbutyl group in pseudo-equatorial position. In iso-octane a mixture of both conformers is present in a ratio such that both CD bands are observed. Although such double curves are often due to vibronic coupling (33), here the two conformers are most probably present, since the two CD bands with opposite sign are also encountered in the region of the K band.

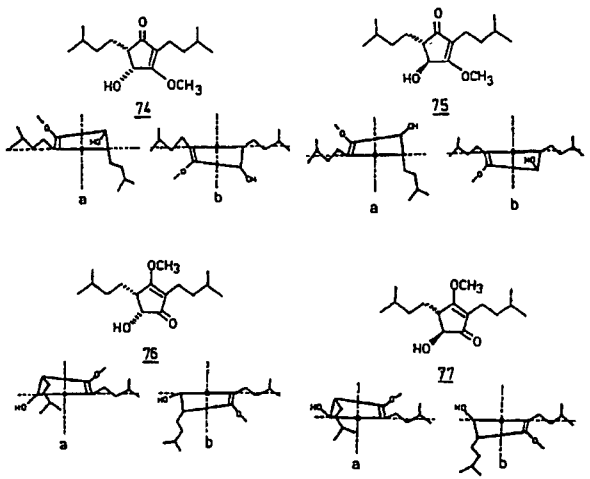


Fig. 41. Octant projections of the methyl enol ethers of the dihydrodeoxohumulinic acids (**74-77**).

Compound **77** occurs in the diaxial conformation (b). The difference with **74** may be explained by the fact that in **74** the small hydroxyl functions are situated next to the methoxy group. In **77** on the other hand this is the isopentyl group, which has less steric interaction in the pseudo-axial conformation. The substituent next to the methoxy group clearly determines the conformational behaviour. In all CD spectra a Cotton effect within the K band is observed. The sign is opposite to that of the R band. A third CD band with a sign opposite to that of the K band is recorded at shorter wavelengths. Comparison of these data with those for the isohumulones **65** and **66** (Fig. 42) allows determination of the enolization pattern. An unusually strong positive Cotton effect is measured for trans isohumulone **66** around 282 nm. This should be assigned to the R band of a β,γ-unsaturated ketone in a conformation, which accounts for interaction between the orbitals of the carbon-carbon and the carbon-oxygen bonds (35). The planes through the carbonyl function and the nodal plane of the double bond should preferably form an angle of 110-120°. This is the case for the β,γ-unsaturated system formed by the carbonyl group of the 4-methyl-3-hexenoyl entity and the double bond of the endocyclic enolized β-triketo system. Indeed, the carbonyl function at C-3 should occur preferably or exclusively in the enol form, since it is very unlikely that the side chain, which contains also a β,γ-unsaturated enone system, prevails in only one helix form. The conformation of **66** may also be deduced from the positive Cotton effect. The pseudo-axial position of the 4-methyl-3-pentenoyl side chain obviates steric interaction with the side chain at C-5. The strong negative R band is in agreement with the chirality of the ring enone system.

The observed positive Cotton effect at 282 nm provides an independent proof for the absolute configuration of **66**. The sign is indeed the same as for the model compound (+) (R)-3-ethanoyl-1,2,3-trimethyl-1-cyclopentene (36). For cis isohumulone (**65**) such Cotton effect for the R band of a β,γ-unsaturated enone would be negative in view of the opposite absolute configuration at C-4. Experimentally, the CD band in this region is also weakly positive. Since the Cotton effects at the longest wavelengths have the same sign and since the enolization is similar in **65** and **66**, the preferred conformation must be the same. The 4-methyl-3-pentenoyl chain prevails in the pseudo-equatorial conformation, hence no particular interaction with the double bond of the ring can take place.

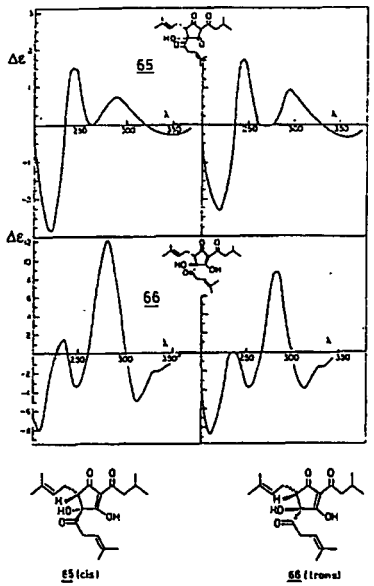


Fig. 42. CD spectra of the isohumulones (65,66).

5.2. FORMATION OF THE ISOHUMULONES.

The isohumulones (65 and 66) are formed by isomerization of humulone (3) in variety of reaction conditions.

5.2.1. BOILING HUMULONE WITH WORT.

This brewing process occurs in the aqueous wort medium, which has a pH value of 5.0-5.5. The reaction is very sensitive to unfavourable conditions and contact with air. The alpha acids utilization yield may be comparable for hops and for hop extracts, but it is usually higher for extracts, because hops have first to be wet and extracted by the wort, before the reaction can start. A low yield may thus be due to the application of

very dry hops or to insufficient stirring. In such cases milling, wetting or ultrasonic treatment of hops may improve the yield. Also, the addition of finely divided material, which may help hop dispersion (Celite), can be favourable. The yield will be higher when pre-isomerized hop extracts are used. The boiling time may in this case be very short. Moreover, iso-alpha acids extracts can be added during fermentation or even to beer after the principal fermentation.

A maximum utilization yield of 50% can be attained by pre-isomerizing hops in alkaline solution and applying a short wort boiling period. Thus, hops could be boiled in a disodium carbonate solution (50 g Na_2CO_3 in 40 l water for 1 kg hops) during 30 min, followed by boiling in wort at a pH value between 5.2 and 5.5 during 30 min. The short boiling time suppresses mainly unwanted side reactions.

In the laboratory this process may be mimicked by application of aqueous buffers with similar pH values, while finely divided material, such as Celite, is added. The purpose is to reproduce the catalytic effect of a large dispersing contact surface, such as the hop leaves and protein precipitate in wort. The concentration of the alpha acids in the brewing kettle is usually around 100-200 $mg.l^{-1}$ at the onset of the boiling, depending on the hop variety and the specific procedure of each brewery (see 1.3.3.). The maximum conversion in the wort boiling stage amounts to 60%, but this value is seldom reached. Large losses occur by insufficient boiling times, inefficient dispersion of hops, oxidative transformations, adsorption on solid material and mainly by the fermentation (38). In practice, a utilization yield of 25-35% (from hop alpha acids to iso-alpha acids in the final beer) is common. The formation mechanism of the isohumulones is identical to that in alkaline conditions.

5.2.2. BOILING HUMULONE IN ALKALINE MEDIA AND IN BUFFERS.

Since the isomerization rate of humulone increases with increasing alkalinity, the isomerization in laboratory conditions is usually performed in alkaline medium. Strong base should be avoided, since the degradation of the isohumulones predominates above a pH value of 12 (see 8.4.1.4.).

The isomerization reaction of humulone to the isohumulones, being the most important reaction in the brewing process, has been studied in great detail (5,8,37). The exact reaction mechanism has been revealed by a thorough investigation of the conditions appropriate for isomerization or epimerization in combination with the knowledge of the relative and absolute configurations (20,39,40) (Fig. 43).

The isomerization requires the intermediacy of the mono-anion of humulone (pK_A

5.5). It follows that the pH values may vary widely. Humulone is a dibasic acid, the strongest acidic function being contained in the β-triketo system, as the corresponding anion is strongly stabilized. Moreover, the β-triketo functionality is responsible for the chelating properties (41) and for the biological activity (42,43). After formation of the anion the remaining enol function in the ring may ketonize in a stereoselective way, yielding the two 3-methyl-2-butenyl side chains in trans position. Thus, an acyloin entity is formed containing a tertiary alcohol function liable to undergo the known α-ketol rearrangement (44). This reaction occurs with ring contraction giving another α-ketol system and a new chiral centre. Consequently, two epimeric compounds, namely cis isohumulone (65) and trans isohumulone (66), are formed by a non-stereoselective ring contraction. The alternative rearrangement of the 3-methyl-2-butenyl group, which would yield an isomeric six-membered ring system, has never been observed. The smooth ring contraction blocks the reaction via the less readily migrating alkenyl chain.

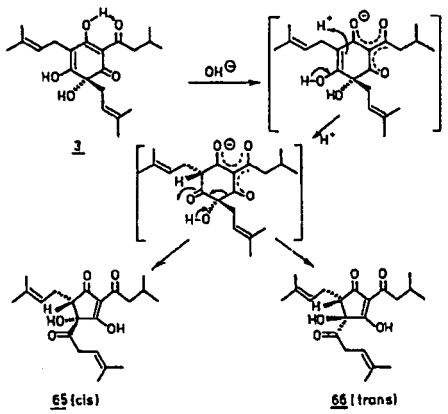


Fig. 43. Mechanism for the isomerization of humulone.

The isohumulones are racemized to the extent of about 15%, which may be due to a partly non-stereoselective protonation upon formation of the intermediate or to epimerization after formation of the isohumulones. Indeed, trans isohumulone can be converted to cis isohumulone at a pH value higher than 9. A true thermodynamic equilibrium can not be reached, because cis isohumulone degrades faster with increasing pH.

Apparently, the composition of the isohumulone mixture depends on the medium in which it is formed. This conclusion contradicts previous ideas (45). Table 9 displays the relative percentages of the isohumulones and the corresponding reaction conditions. The ratio trans isohumulone : cis isohumulone of 32:68 is the composition in normal brewing conditions. Cis isohumulone is the thermodynamically most stable compound, since the two large vicinal side chains are in the trans configuration.

Table 9. The percentages of the isohumulones (%) in different reaction conditions.

Reaction conditions	Trans isohumulone (%)	Cis isohumulone (%)
5.5	32	68
7.0	32	68
9.3	34	66
11.05	36	64
NaOH 0.1 N	41	59
NaOH 2 N (92°C)	45	55
NaOH 2 N (50°C)	49	51
NaOH 1N + $MgSO_4$	45	55
Iso-octane : Na_2CO_3 0.3 N	30	70

The considerable increase of the isomerization rate, caused by a divalent cation such as magnesium (46) or by strong alkali, is due to the formation of the di-anion (47). The isomerization, catalyzed by magnesium(II) ions, is very remarkable. The conversion proceeds with a yield of at least 90% within 10 min at 70°C and the ratio cis isohumulone : trans isohumulone is 55:45 (48).

Isomerization of humulone.

a) In aqueous buffer solutions.

Humulone (2 g; 5.52 x 10^{-3} mol) is dissolved in ethanol (10 ml) and an aqueous buffer solution (3.5-4 l). After boiling under nitrogen during 1/4-2 h, the solution is cooled, acidified with HCl 1 N to pH 1 and extracted with iso-octane or ether (4 x 1 l). The combined extracts are washed with water (250 ml), dried ($MgSO_4$) and concentrated in vacuo. Some examples of buffer solutions are as follows:

- disodium carbonate (pH 10.9) : 1/4 h;

- phosphate (pH 8.9) : 1.1/2 h;

- phosphate - citric acid (pH 7.0) : 1.3/4 h;

- sodium citrate - citric acid (pH 5.4) : 2.1/2 h.

The concentration of humulone is similar to that in brewing conditions. However, more concentrated solutions may be applied. For example, humulone (20 g; 5.52 x 10^{-2} mol) is boiled in an aqueous phosphate buffer (2 l) with pH 11 under nitrogen during 1.5 h (49). The yields are 60-80% depending on the particular reaction conditions.

b) In a two-phase system of iso-octane : disodium carbonate.

Humulone (0.7 g; 1.93 x 10^{-3} mol) is dissolved in iso-octane (20 ml) and disodium carbonate solution (0.3 N; 20 ml). After boiling during 30 min, the solution is cooled, acidified with cold HCl 1 N and extracted with iso-octane. The extract is dried and concentrated in vacuo. The yield of isohumulones is 85%.

c) In alkali, catalyzed by magnesium(II) ions.

Magnesium sulfate heptahydrate (21.5 g; 8.74 x 10^{-2} mol) is dissolved in water (250 ml) and methanol (300 ml). At 70°C are added : methanol (500 ml), sodium hydroxide (1 N; 52.5 ml) and humulone (18 g; 4.97 x 10^{-2} mol). After 10 min the solution is cooled and worked up as described under b) (54,56).

5.2.3. PHOTO-ISOMERIZATION OF HUMULONE.

It had been observed that the UV spectrum of a methanolic humulone solution, exposed to light, changes over a period of 28 days to match that of the isohumulones (49). In a more detailed study a 0.2% methanolic solution of humulone was irradiated with a high-pressure mercury lamp during 36 h (50). A crystalline substance was obtained after removal of the solvent and recrystallization of the residue from iso-octane. This so-called photo-isohumulone was identical to trans isohumulone (68). The photoreaction also proceeds upon irradiation with UV light of 365 nm or 254 nm. The presence of oxygen and the nature of the alcoholic solvent are irrelevant (51). The quantum yield, measured by ferrioxalate actinometry at 350 nm, is 0.022 (52). Since no

trace of cis isohumulone 65 has been detected in the reaction mixture, it is clear that the photorearrangement occurs in a totally regioselective and stereoselective way. Experimentally it is very difficult to identify unequivocally the multiplicity of the reactive excited state for several reasons :

- the extremely weak luminescence of the hop bitter acids, thereby eliminating luminescence studies ;
- the high quencher concentration needed to observe a small amount of quenching in view of the short lifetime of the reactive state;
- the difficulties in the interpretation of sensibilization experiments due to tail absorption of humulone into the visible region.

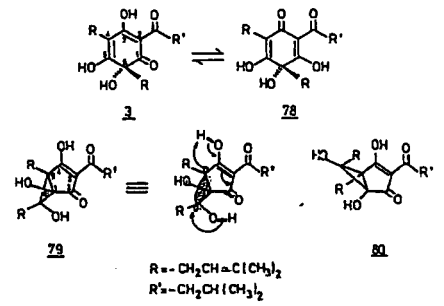


Fig. 44. Structural formulae of the humulone tautomers (3,78) and the possible photo-isomerization products (79,80).

The reaction could proceed via the known lumiketone rearrangement of the tautomeric 2,5-cyclohexadienone (78, Fig. 44) (54). This is however not the case. Since the equilibrium between humulone and 78 is shifted in favour of tautomer 3, all the light is absorbed by 3. Photo-enolization of 3 would only lead to the ground state of 78 (55). It may be concluded that the rearrangement proceeds via the known dienol form of humulone (3, Fig. 44). Usually, 2,4-cyclohexadienones are cleaved photochemically in the α-position yielding a diene-ketene with concurrent loss of the

stereochemical integrity at the α-carbon atom (56). This mechanism has to be excluded since racemization has not been observed.

The reaction can however take a different course when the irradiation is carried out in polar medium (57) or when the substrate is heavily substituted (56,57). In this case a product analogous to that formed upon the lumiketone rearrangement is found as a result of a (1,2)-acyl shift, the so-called oxa-di-π-methane rearrangement (ODPM) (58). The abovementioned requirements, which are both fulfilled for the humulone rearrangement, decrease the energy of the (π,π^*) excited singlet state with respect to the (n,π^*) state. The reactivity difference may be explained by invoking the intermediacy of a (n,π^*) state for the ketene formation and a (π,π^*) state for the ODPM rearrangement.

The ODPM rearrangement can be considered as a concerted $(\pi^2 + \pi^2)$ cycloaddition reaction between the C-6/C-1 σ-bond and the C-4/C-5 π-bond. The reaction is photochemically allowed when both bonds react either in a suprafacial or in an antarafacial manner (59). The suprafacial reaction is impossible since a trans coupled bicyclo[3.1.0]hexenone system would result. The antarafacial reaction implies inversion at C-6 (60-62). Since the addition to the double bond can occur both from the upper face and from the lower face, two diastereomeric bicyclo[3.1.0]hexenone derivatives 79 and 80, respectively, may be formed (Fig. 44). These intermediates can not be isolated because of the immediate cyclopropanol rearrangement (63). Although each one of the cyclopropane bonds may be broken, only the C-5/C-6 bond is cleaved thereby accounting for the regioselectivity.

This reaction course is caused by an internal electrophilic substitution with the enolic hydrogen atom acting as the electrophile. The geometry is such that the proton is attacking along the σ-bond being broken. In such cases inversion at the reaction centre is observed (63), while retention of configuration usually occurs when the electrophile reacts in an edge-on fashion (64-66). This explains the stereoselectivity of the reaction.

The smooth regio- and stereoselective ring cleavage is undoubtedly to be ascribed to the pericyclic character of the rearrangement within a Hückel-system (79, Fig. 44). This is confirmed by the different behaviour of lupulone in similar conditions, whereby a 3-methyl-2-butenyl group replaces the hydroxyl function at C-6 in humulone (see 3.4.). Only intermediate 79 is in agreement with the experimental stereochemical

results. Indeed, the cyclopropanol rearrangement of 80 would lead to the enantiomer of 79 with concurrent racemization. Similar selectivity upon formation of bicyclo[3.1.0]hexenones from photo-isomerization of alkyl substituted cyclohexa-2,5-dienones has been reported (67). It has been shown that the stereochemistry is controlled mainly by steric factors, the stereochemical control being exerted by the pseudo-equatorial alkyl group at C-6, which is in an exo position in 79 and in an endo position in 80.

The ODPM rearrangement is stereoselective with complete inversion at C-6. It is regioselective by the exclusive formation of the intermediate 79. The cyclopropanol rearrangement of 79 proceeds in a regioselective way with total inversion at the reaction centre, while cleavage of only one cyclopropane bond accounts for the regioselectivity.

Photo-isomerization of humulone.

Humulone (2 g; 5.52 x 10^{-3} mol), dissolved in methanol (200 ml), is irradiated with light of either 254 nm or 350 nm under nitrogen. After 10-12 h the solvent is removed and the residue is recrystallized from iso-octane. The white crystals are identified as trans isohumulone (melting point 65°C). The reaction time is proportional to the amount of substrate.

5.2.4. THERMAL ISOMERIZATION OF HUMULONE.

In the absence of oxygen, humulone resists heating up to 100°C (66). On the other hand, trans isohumulone is converted under these conditions to humulone (5-10% after 2 h). Above 180°C the thermal transformations and degradations of humulone occur very rapidly yielding complex reaction mixtures. Both in the solid state and in a solvent, such as dimethyl sulfoxide (boiling point 196°C), a mixture of isohumulones in a cis : trans ratio of 85:15 is produced. Since these compounds are degraded very quickly, a maximum yield of only 5% can be obtained. At the high reaction temperatures both isohumulones are mutually converted by epimerization, possibly via humulone, which indeed can be traced back in the thermal reaction mixtures derived from the isohumulones. Such epimerization occurs also in alkaline medium (see 5.3.). Furthermore, cis isohumulone is degraded 3.6 times faster than trans isohumulone. It is therefore not clear whether or not cis isohumulone is formed exclusively upon thermal treatment of humulone.

Table 10. Thermal treatment of metal humulates.

Metal humulate	Result
Aluminum(III)	No reaction
Antimony(III)	Traces of isohumulones
Barium(II)	Partial conversion
Cadmium(II)	Almost only isohumulones
Calcium(II)	Only isohumulones
Cerium(III)	Partial reaction
Chromium(III)	No change
Cobalt(II)	Only isohumulones
Copper(II)	Degradation, no isohumulones
Iron(II)	Degradation products, isohumulones
Iron(III)	Strong degradation
Lead(II)	Only humulone
Lithium(I)	Complete conversion to the isohumulones
Magnesium(II)	Only isohumulones
Manganese(II)	Isohumulones, degradation products
Mercury(II)	Strong degradation
Nickel(I)	Complete conversion to isohumulones
Potassium(I)	Partial reaction
Sodium(I)	Partial reaction
Strontium(II)	Partial reaction
Tin(II)	Partial reaction
Zinc(II)	Isohumulones, humulone

The isomerization proceeds most efficiently upon heating of the solid metal salts of humulone, which are precipitated first from aqueous alkaline solutions (68). The results are dependent on the nature of the metal ions (Table 10). Heating is achieved at 110°C during 1 h. Some metal salts, such as those derived from aluminum(III), chromium(III) and lead(II) are stable, while the copper(II) salts and the mercury(II) salts undergo rapid degradation. The iron humulates are isomerized partially with concurrent degradation. The following salts are isomerized without degradation : antimony(III), barium(II), cadmium(II), cerium(III), potassium(I), sodium(I), strontium(II), tin(II) and zinc(II) humulates.

Further heating of the potassium and sodium salts gives better yields. It appears that isohumulates derived from metal ions, which may occur in different oxidation states, are unstable (69). The best results are obtained with the cobalt(II) and the nickel(II) salts, but particularly with the calcium(II) and magnesium(II) humulates, which are commercially important in the production of isomerized hop extracts (70). The high yield and the purity of the reaction product are of great importance. The lithium(I) and manganese(II) salts are less appropriate. Patents have been granted for isomerization of alkaline earth metal salts of humulone in an organic solvent in the presence of other hop components (71,72).

Thermal treatment of metal humulates.

An aqueous alkaline solution of potassium humulate is obtained by counter-current extraction of a trichloroethylene extract of fresh hops in a column packed with aqueous dipotassium carbonate (0.055 M). Upon saturation with potassium chloride, potassium humulate is precipitated. The supernatant is decanted and the solid material is isolated.

The sodium and lithium humulates are obtained as follows. After acidification of the aqueous potassium humulate extract (100 ml) with HCl (4 N; 10 ml), the emulsion is extracted with iso-octane (2 x 50 ml). The combined extracts are washed with water (3 x 50 ml) and extracted with a disodium or dilithium carbonate solution (0.055 M; 90 ml). The carbonate extract is saturated with sodium or lithium chloride, as described before.

The water-insoluble metal humulates are prepared by addition of water-soluble salts of the corresponding metals (1.05 molar equivalents) to an aqueous potassium humulate extract (100 ml). After mixing, the precipitated metal humulates are isolated by filtration. The solid material contains 60-80% water.

The metal humulate is heated at 110°C during 1 h. After cooling the aqueous phase is separated and the solid residue is analysed. The results given in Table 10 were obtained by thin layer chromatography as follows. The plates are prepared with a suspension of silica gel G in a 1% aqueous phosphoric acid solution. After activation at 100-110°C during 1.5 h, the plates are developed with MeOH : HCl 9:1 to remove interfering impurities, followed by drying at 100-110°C during 30 min. Detection is done by spraying with a solution of iron(III) chloride.

The quantitative character of the results may be questioned. Today these analyses would be achieved by LC.

5.2.5. ISOMERIZATION OF HUMULONE IN ALKALINE METHANOL. SPIRO-ISOHUMULONES.

5.2.5.1. ISOLATION AND IDENTIFICATION.

The isomerization of humulone in alcoholic solution is quite remarkable and not at all comparable with the reaction in brewing conditions. Carson (73) obtained a number of crystalline fractions upon heating humulone during 3-6 h in methanolic potassium hydroxide or sodium methylate. The structure of the reaction products has been investigated thoroughly (8,47), but the purification of the fractions was not straightforward (74,75). Complete separation was achieved by preparative thin layer chromatography on Kieselgel with xylene : chloroform : isopropanol 5:4:1 as eluent. Previous concentration can be done by counter-current distribution in the two-phase system carbon tetrachloride : 50% aqueous formamide. The mixture is only partially separated after 2000 transfers.

Four isomeric compounds 81-84 (Fig. 45) have been isolated with Rf-values of 0.61, 0.47, 0.35 and 0.25, respectively, in relative ratios of 50:7:41:2. The compounds have a spiro structure and are isomeric with the isohumulones. Consequently, these so-called Carson isomers have been designated as spiro-isohumulones (76,77). The rules for the assignment of cis-trans isomers are valid (13).

The similarity with the isohumulones follows from the almost identical UV spectra in acidic and in alkaline medium and from the comparable pK_A values. Only dihydroderivatives can be prepared. The dihydrocompound of 81 has a melting point of 139-142°C and a $[\alpha]_D$-value of +119 in methanol. The corresponding derivative of 83 melts at 150-152°C and has a specific optical rotation at the Na_D-line of -75.5. Alkaline degradation of the spiro-isohumulones leads to the humulinic acids (see 8.4.1.) in 90% yield, while the dihydroderivatives give rise to the dihydrohumulinic acids (see 8.4.3.1.). It is clear that the double bond of the 4-methyl-3-pentenoyl side chain of the isohumulones is involved in the formation of the spiro-isohumulones. This is confirmed by acidic or light alkaline treatment, whereby about 10% isohumulones are formed. As such, the reversibility of the conversion of the isohumulones to the spiro-isohumulones is demonstrated.

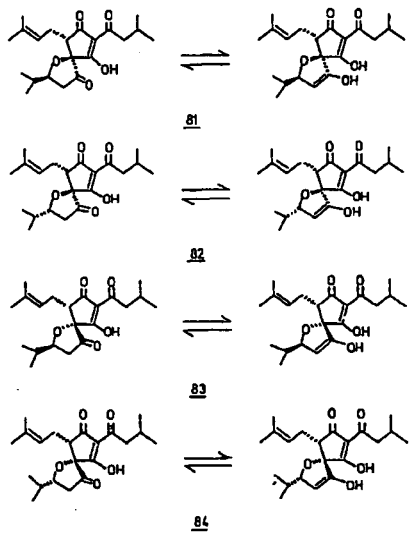


Fig. 45. Structural formulae of the spiro-isohumulones (81-84).

The IR and mass spectra are very similar for all four structures. The absence of dehydration in the mass spectrum indicates that the alcohol function at C-4 in the isohumulones is masked in the spiro-isohumulones. No fragment-ion is observed corresponding to the cleavage of the 4-methyl-3-pentenoyl group at C-4. The ^1H NMR data show that 81 and 82 belong to the trans series, while 83 and 84 are cis compounds. This follows from the complexity of the spectra (see 5.1.3.1.). Thus, at least two pairs of doublets are found for the geminal dimethyl groups of the 3-methylbutanoyl side chain for compounds 81 and 82. The olefinic triplet around δ 5 integrates for only one proton and the typical doublet for the methylene protons of the 4-methyl-3-pentenoyl side chain around δ 3.2-3.4 is absent. Furthermore, absorptions

are found around δ 4, due to a methine proton next to a carbon-oxygen single bond. These data prove the spirobicyclic structure, which prevails for about 40% in the enol form. This feature is shown by the doublet at δ 4.68, accounting for 0.4 proton and by the doublet at δ 1.6, which is caused by methyl groups on a double bond. Also, in other regions of the spectrum double signals are observed, although these are less clearly separated. The protons of the enol form are shifted to higher field. It is known that tetrahydrofuran-3-one derivatives are enolized depending on the position and the nature of the substituents and on hydrogen bridge formation (78,79).

5.2.5.2. FORMATION MECHANISM.

As an alternative to the deacylation of the isohumulones in aqueous alkaline medium (see 5.2.2.) an intramolecular Michael addition occurs in alcoholic alkaline medium, following the shift of the double bond. The allo-isohumulones (see 8.1.) are the most likely intermediates in the formation of these 2'-(3-methylbutanoyl)-4'-(3-methyl-2-butenyl)-2-(1-methylethyl)-1'-hydroxy-1-oxaspirobicyclo[4.4]non-1'-ene-4, 3'-diones or so called spiro-isohumulones. In addition to the two existing chiral centres a new chiral centre is created by the internal cyclization reaction. As a consequence four diastereo-isomeric compounds are isolated. The distinction between the cis stereo-isomers follows from the ^{1}H NMR characteristics, based upon the greater complexity of the more hindered structures (21). A complicating factor for the spiro-isohumulones is the spatial position of the isopropyl group on the heterocyclic ring. Experimentally, a triplet is found for the ring methine proton both for the cis and the trans compounds. On the contrary, the sp^2-protons display a more complex pattern for the minor compounds 82 and 84. This feature indicates that the chirality, introduced upon cyclization to the furanone structure, is more pronounced in 82 and 84 compared to 81 and 83. This behaviour has to be ascribed to the isopropyl group at the new chiral centre, which is directed towards the 3-methyl-2-butenyl side chain thereby creating steric hindrance. The difficult formation of these isomers is reflected in the low yield. Compounds 81 and 82 are the exo and endo trans spiro-isohumulones, respectively, while 83 and 84 represent the exo and endo cis spiro-isohumulones, respectively.

The bitterness and the water-solubility of the spiro-sohumulones are lower than those of the isohumulones. They do not occur in aqueous isomerization mixtures of humulone.

Preparation and separation of the spiro-isohumulones.

Humulone (10 g; 2.76 x 10^{-3} mol) is boiled during 30 min in methanol (80 ml) containing potassium hydroxide (3.56 g; 6.36 x10^{-2} mol). Acidification, extraction with ether and removal of the solvent give a residue, which is extracted with iso-octane. The insoluble fraction (1.8 g) is a mixture of the spiro-isohumulones. Preparative thin layer chromatography plates (20 x 40 cm; d : 1.25 mm; Kieselgel Merck PF-254) are dried at 120°C during 3 h. The separation is performed with samples of 0.08-0.1 g mixture per plate, in the eluent xylene : chloroform : isopropanol 5:4:1. Four zones are detected under UV light with Rf values of 0.61 (50%), 0.47 (7%), 0.35 (41%) and 0.25 (2%), respectively. After extraction of the four fractions with acetone, the residues are recrystallized from benzene : iso-octane or from n.propanol after removal of the solvent. The respective melting points and the optical rotations at the Na_D-line in methanol are :

81 : 123.5-124.5°C; +110.3
82 : 99-101°C; +5
83 : 124.5-125.5°C; -60.1
84 : 138-140°C.

The UV absorption maxima are at 240 (ε 11600) nm and 277 (ε 10200) nm in MeOH : HCl 0.1 N; 246 (ε 19800) nm and 270 (ε 16000) nm in MeOH : NaOH 0.1 N.

Counter-current distribution in the two-phase system carbon tetrachloride : 50% aqueous formamide gives partial separation after 2000 transfers. These enriched fractions are suitable for preparative thin layer chromatography.

5.3. INTERCONVERSION BETWEEN ISOHUMULONES AND HUMULONE.

Epimerization of the isohumulones, i.e. the interconversion of cis into trans isohumulone and vice versa, proceeds via inversion of one chiral centre. This process can only be studied in aqueous alkaline medium, since interfering reactions occur in acidic conditions. As the base becomes stronger, degradation also increases (see 8.4.1.4.). The optimum pH value is around 11.0. In these conditions the degradation rate of cis isohumulone is faster than the epimerization rate. In trans isohumulone the epimerization rate (half-lifetime 380 min) is faster than the degradation rate. Epimerization at C-5 can occur directly via the anion in α-position with respect to the carbonyl group. In this case the enantiomer of the commonly found cis isohumulone is formed. On the other hand, epimerization via the quaternary ring carbon atom C-4 can only occur via ring expansion to humulone, which then gives rise to the mixture of

isohumulones.

In practice, the isohumulones can indeed be converted to humulone, since the compounds all possess an acyloin entity and the acyloin rearrangement is reversible. Up to 2% humulone can be isolated by isomerization of trans isohumulone in aqueous buffers of pH 10.0 and 11.0 (80). *The yield of humulone would even amount to 10%*, when cis isohumulone is shaken in the two-phase system iso-octane : aqueous buffer pH 5.0 (81,82). It has been found that the conversion of the isohumulones to humulone occurs between pH values of 2.5 and 12.0 (66,83). The same phenomenon is found upon heating to 100°C *with a maximum yield* of 15% after 20 h.

Further heating causes decomposition. Consequently, the isohumulones can never be obtained in pure form by distillation because of humulone impurities. It can not be concluded whether traces of humulone, present in beer (84), *are due to non-reacted humulone* from hops or to conversion of the isohumulones. The ring expansion most probably follows the same stereochemical pathway as the ring contraction, which is reflected in the degree of racemization (about 15%) for both reactions. It is found *that cis isohumulone, obtained from trans* isohumulone, is racemized for 45% in agreement with the reaction sequence humulone - trans isohumulone - humulone - cis isohumulone. Trans isohumulone obviously epimerizes to cis isohumulone via humulone in aqueous buffer solutions up to pH 12.0. *Isomerization via the carbanion* at C-5, which accounts for the epimerization of the humulinic acids (see 8.4.1.4.), may occur in stronger alkaline medium, but it is then competitive with degradation reactions.

It is remarkable that an equilibrium exists between the *humulates and the isohumulates* of zinc(II) and lead(II) (69). Thus about 10% zinc(II) humulate is formed from zinc(II) isohumulate. Heating of lead(II) isohumulate at 110°C gives after 2 h only the lead(II) humulate, while degradation occurs after prolonged reaction time. This isomerization, in optimized conditions, *could give rise to conversion* of the isohumulones to humulone in high yield.

5.4. SYNTHESIS OF THE ISOHUMULONES.

Racemic isohumulone is prepared from the key product 2-methyl-2-penten-4-yne, which is obtained by a 1,4-elimination reaction from 1-bromo-4-methylpenta-1,2-diene, in the presence of copper(I) cyanide (85). Addition of the hydrocarbon to ethyl pyruvate, followed by hydrolysis, leads to 2,6-dimethyl-2-hydroxy-5-hepten-3-yne acid. *The corresponding* acid chloride is added to ethyl 3-oxo-5-methylhexanoate in the presence of magnesium methylate. The reaction product cyclizes in basic medium to

2-(3-methylbutanoyl)-3,4-dihydroxy-4-(4-methyl-3-penten-1-ynyl)-2-cyclopentenone. Alkenylation with 1-bromo-3-methyl-2-butene in the presence of sodium methylate, followed by hydration of the triple bond, gives rise to racemic isohumulone (Fig. 46).

The overall yield, starting from 2-methyl-2-penten-4-yne, amounts to only 0.07%. In an analogous way racemic isocohumulone and isoadhumulone are obtained but again in very low yield (86). The corresponding reagents are then ethyl 4-methyl-3-oxopentanoate and ethyl 4-methyl-3-oxohexanoate for isocohumulone and isoadhumulone, respectively.

Synthesis of the isohumulones.

1-Bromo-4-methylpenta-1,2-diene.

A stirred mixture of *copper(I) bromide* (66 g; 4.6×10^{-1} *mol*), *ammonium bromide* (45 g; 4.6×10^{-1} mol), copper powder (3 g; 4.7×10^{-2} mol) and hydrogen bromide (60 volume %; 180 ml) is cooled to 5°C and subsequently treated with 4-methylpent-1-yn-3-ol (49 g; 5×10^{-1} mol). The mixture is stirred during 1/2 h and *subsequently heated to* 45°C for 3/4 h. After cooling, extraction with petroleum ether, washing (60% ammonium bromide and water), drying and removal of the solvent, the residual oil is distilled under nitrogen to yield the 1-bromo-4-methylpenta-1,2-diene as a light-yellow oil (58.1 g; 79%) with a boiling point of 44-46°C/8 mm Hg.

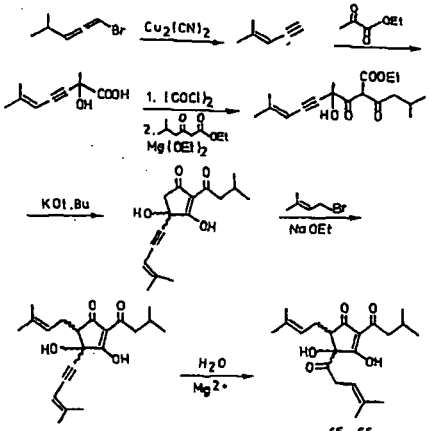


Fig. 46. Synthesis of the (racemic) isohumulones. 65, 66

2-Methyl-2-penten-4-yne.

A mixture of 1-bromo-4-methylpenta-1,2-diene (20 g; 1.24 x 10^{-1} mol) and dry copper(I) cyanide (14 g; 1.56 x 10^{-1} mol) is heated in a distillation vessel. After about 1/4 h white fumes are evolved and a fraction with boiling point of 29°C is collected. The temperature is raised to 79-80°C and a second fraction (7-8 ml) is obtained. Subsequently, a vigorous reaction is observed, while no more product can be collected. Dilution of the distillate with water and extraction with ether give, after washing with water, drying and concentration in vacuo, 2-methyl-2-penten-4-yne (5.2 g; 52%) as a colourless liquid with a boiling point of 79-80°C/759 mm Hg.

UV : λmax (ε) : 220 (10050) nm in ethanol.

IR : vmax : 3310, 2980, 2900, 2100, 1630, 1440, 1380, 1330, 1195, 1080, 975, 820 cm^{-1}.

Ethyl 2-hydroxy-2,6-dimethyl-5-hepten-3-ynoate.

A stirred solution of magnesium wire (2.4 g; 0.99 mol) in dry tetrahydrofuran (20 ml) is added dropwise to a solution of 1-bromopropane (12.3 g; 0.1 mol) in dry tetrahydrofuran (15 ml). After activation with iodine the mixture is boiled during 1 h, cooled to 0°C and treated with a solution of 2-methyl-2-penten-4-yne (10 g; 1.25 x 10^{-1} mol) in dry ether (10 ml). Efficient stirring is necessary since the Grignard reagent is only partially soluble. Upon heating to 25°C during 0.5 h propane evolves violently. After boiling during 2 h and cooling to 0°C, a solution of ethyl pyruvate (11.6 g; 0.1 mol) in dry tetrahydrofuran (20 ml) is added. The mixture is boiled for 3-4 h, cooled to 0°C and hydrolyzed by adding hydrogen chloride (2 N; 70 ml). Dilution with ether, washing with water, drying and concentration in vacuo give a dark-red oil, which leads, after distillation, to ethyl 2-hydroxy-2,6-dimethyl-5-hepten-3-ynoate (8.7 g; 44%) with a boiling point of 60°C (bath temperature)/2.7 x 10^{-2} mm Hg.

UV : λmax (ε) : 235 (9280) nm.

IR : vmax : 3450, 3000, 2220, 1790, 1440, 1380, 1250, 1210, 1135, 1100, 1010, 940, 860, 820 cm^{-1}.

2-Hydroxy-2,6-dimethyl-5-hepten-3-yne acid.

A solution of ethyl 2-hydroxy-2,6-dimethyl-5-hepten-3-ynoate (5.0 g; 2.55 x 10^{-2} mol) in methanol (25 ml) is added to a solution of sodium hydroxide (15 g; 3.75 x 10^{-1} mol) in methanol (100 ml) and kept at room temperature for 72 h. The mixture is diluted

with water (150 ml), cooled to 0°C and acidified with hydrogen chloride (2 N; 85 ml) under nitrogen. The solution is extracted with ethyl acetate and the organic layer is extracted with saturated aqueous sodium hydrogen carbonate. Acidification (HCl), extraction with ethyl acetate, washing with water, drying and concentration in vacuo give 2-hydroxy-2,6-dimethyl- 5-hepten-3-yne acid (2.6 g; 61%) with a boiling point of 85°C (bath temperature)/8 x 10^{-3} mm Hg.

UV : λmax (ε) : 236 (10050) nm.

IR : vmax : 3450, 2980, 2210, 1720, 1440, 1380, 1320, 1220, 1140, 940, 870, 815, 730 cm^{-1}.

2-Hydroxy-2,6-dimethyl-5-hepten-3-ynoyl chloride.

A stirred solution of the acid (1.6 g; 0.95 x 10^{-2} mol) in dry ether (25 ml) is cooled to 0°C and a solution of oxalyl chloride (1.25 g; 0.98 x 10^{-2} mol) in dry ether (10 ml) is added dropwise. The mixture is further stirred for 18 h at room temperature. Volatile components are removed in vacuo at 25°C, whereby 2-hydroxy-2,6-dimethyl-5-hepten-3-ynoyl chloride (1.85 g; 100%) remains as a light-brown oil.

2-(3-Methylbutanoyl)-3,4-dihydroxy-4-(4-methyl-3-penten-1-ynyl)-2-cyclopentenone.

To a suspension of magnesium methylate (0.35 g; 3.06 x 10^{-3} mol) (86) in dry benzene (50 ml) is added a solution of ethyl 5-methyl-3-oxohexenoate (1.7 g; 9.88 x 10^{-3} mol) (88) in dry benzene (50 ml). The mixture is boiled for 0.5 h, cooled and treated dropwise with a solution of the acid chloride (1.86 g; 9.97 x 10^{-3} mol) in dry benzene (5 ml). This mixture is boiled (0.5 h), cooled to room temperature and stored (18 h). Acidification (HCl), dilution with ether, washing, drying and removal of the organic phase lead to the crude product as a brown oil (3.2 g). Chromatography on silica gel (25 g per g product) with ethyl acetate : hexane 3:17 as eluent gives yellow oily (0.080 g from 1 g crude product) 6-ethoxycarbonyl-6-hydroxy-2,6,11-trimethyl-2-dodec-4-yne-7,9-dione (5%). A solution of this ester (0.5 g; 1.55 x 10^{-3} mol) in dry toluene (50 ml) and dry t.butanol is treated with potassium t.butylate (1.0 g; 8.93 x 10^{-3} mol) and boiled for 24 h. After cooling, acidification with dilute HCl and extraction with ether, the ether layer is separated, washed, dried and concentrated in vacuo. The residual brown oil (0.38 g) is chromatographed on silica gel with ethyl acetate as eluent. Distillation yields 2-(3-methylbutanoyl)-3,4-dihydroxy-4-(4-methyl-3-penten-1-ynyl)-2-cyclopentenone (0.053 g; 12%) with a boiling point of 70°C (bath temperature) at 5 x 10^{-4} mm Hg.

UV : λmax (ε) : 271 (23880) nm in alkaline ethanol.

IR : vmax : 3450, 3000, 2260, 1740, 1650, 1470, 1370, 1230, 1180, 1040, 900, 745 cm^{-1}.

2-(3-Methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(4-methyl-3-penten-1-ynyl)-2-cyclopentenone.

The cyclopentane dione (0.154 g; 5.58 x 10^{-4} mol) in dry ether (5 ml) is added to a vigorously stirred solution of sodium (0.3 g; 1.3 x 10^{-2} mol) in dry ethanol (10 ml). After cooling to 0°C a solution of 3-methyl-2-butenyl bromide (0.075 g; 5.03 x 10^{-4} mol) in ether (5 ml) is added. The mixture is stirred for 18 h, diluted with water, acidified (HCl) and extracted with ether. The ethereal layer is separated, washed with water, dried and concentrated in vacuo to an oil. Distillation yields 2-(3-methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(4-methyl-3-penten-1-ynyl)-2-cyclopentenone (0.084 g; 44%) as a light-yellow oil with a boiling point of 100°C (bath temperature) at 5 x 10^{-4} mm Hg.

UV : λmax (ε) : 285 (15020) nm in alkaline ethanol.

IR : vmax : 3450, 3000, 2260, 1800, 1720, 1600, 1450, 1380, 1180, 1110, 1020, 890, 760 cm^{-1}.

Racemic isohumulones.

To a stirred solution of mercury(II) sulfate (0.075 g; 2.53 x 10^{-4} mol) in methanol (5 ml), containing 50% dihydrogen sulfate (1 ml), is added dropwise a solution of the reaction product from the previous procedure (0.075 g; 2.18 x 10-4 mol) in methanol. The mixture is heated to 40°C and stirred for 4 h. After cooling, dilution with water and extraction with ether, the ethereal solution is washed with water, dried and concentrated in vacuo to a yellow oil (0.062 g). Thin layer chromatography on silica gel under nitrogen with hexane : ethyl acetate 85:15 as eluent indicates the presence of the isohumulones. The appropriate zone is eluted with methanol : water 1:1 and the resulting solution is diluted with ether, dried and concentrated in vacuo. The residue is distilled to give a yellow oil (0.015 g; 19%) with a boiling point of 110°C (bath temperature) at 5 x10^{-4} mm Hg. The spectrometric characteristics are claimed to be fully in agreement with those of the natural isohumulones.

5.5. IMPORTANCE OF THE ISOHUMULONES.

The isohumulones, together with the co-, ad-, pre- and postisohumulones, constitute the beer iso-alpha acids mixture, which is formed upon boiling of hops with

wort in the brewery. These iso-alpha acids represent more than 80% of all hop components occurring in beer. The concentration varies from 15 to 80 $mg.l^{-1}$. The beer iso-alpha acids are responsible for the typical bitter taste of beer. The bitter character does not differ very much among the iso-alpha acids (89). The readily available pure crystalline trans isohumulone (see 5.2.3.) is usually applied as reference compound in taste trials involving hop derivatives. Unhopped beer, to which 30 mg.l-1 trans isohumulone is added, is almost as bitter as hopped beer, containing 25 $mg.l^{-1}$ iso-alpha acids. Nevertheless, subtle taste differences cause a definite albeit not a generally shared preference for hopped beer among taste panel members. An explanation is not readily available.

As a quantitative criterion for the degree of bitterness the European Bitterness Unit (EBU) is accepted by the European Brewery Convention (90-93). These EBU values are obtained by measuring the absorbance of a beer iso-octane extract at 275 nm, the absorption maximum of the iso-alpha acids. In practice this value is determined as follows : 10 ml degassed beer is acidified with 1 ml HCl 3 N and extracted with 20 ml iso-octane. Subsequently, the absorbance of the extract is measured at 275 nm in a quartz tube of 10 mm length; the bitter degree according to the EBC norms is found by application of the formula :

EBU = A(275 nm) x 50

It is obvious that this absorbance includes other beer-extractable components, which absorb at 275 nm, in addition to the isohumulones. It is well established that iso-octane extracts about twice the weight from beer than the amount of iso-alpha acids present. The error in the absorbance measurement is, however, probably small, since these other components consist mainly of oxidized fatty acids (precursors of the sunstruck flavour, 15 to 20 $mg.l^{-1}$ in lager beer) and other, non-UV active substances.

The iso-alpha acids have tensioactive properties which stabilize beer foam. This effect was discovered in 1916 (94) and has been confirmed repeatedly (95-101). In particular, complexes of the iso-alpha acids with some metal ions are important (102). The concentration of the iso-alpha acids in the foam head is always higher than in the bulk of the beer, but when nickel(II) ions are added this effect is enhanced (103,104). Also, there is a clear relationship between the stability of the beer foam after addition of nickel(II) ions and the iso-alpha acids content of beer (105). It has been shown that cobalt(II) ions increase the foam stability (106). Other metal derivatives of the isohumulones are less active in this respect (107). Furthermore, nickel(II) ions may be

related to the gushing of beer (108). At one time the lethal toxic effects of beer, to which cobalt(II) salts had been added to stabilize the foam, was front page news.

The various facets of beer foam formation have been described by Bishop (101,108). The increased concentration of the iso-alpha acids in the foam could possibly lead to precipitation of metal isohumulates (in particular those of iron(II), nickel(II), cobalt(II) and zinc(II)), whereby the film around the air bubbles would be strenghtened leading to increased foam stability. This effect is probably reinforced by formation of complexes between the iso-alpha acids or their salts and proteins. It appears that such complexes only exist at sufficiently high concentration, i.e. at the surface of small air bubbles, when the beer starts foaming. The foam is stabilized by the tensio-activity of the complexes, which is more pronounced compared to that of the free acids (109).

The iso-alpha acids inhibit the growth of Gram-positive bacteria and therefore protect beer against micro-organisms (110) (see 1.3.2.).

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CHAPTER 6
REDUCED DERIVATIVES OF THE ISOHUMULONES

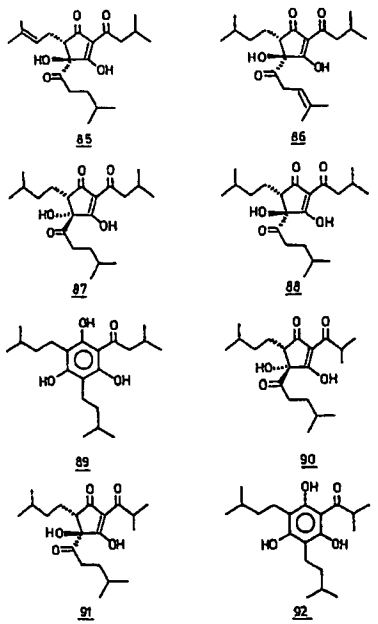
General remarks on the reduction of the hop bitter acids, are given in the first *section of Chapter 3.*

6.1. DIHYDRO-ISOHUMULONES.

Hydrogenation of trans isohumulone with Adams' catalyst in aqueous disodium carbonate affords a resinous oil. Trans dihydro-isohumulone (1), (molecular formula $C_{21}H_{32}O_5$) may be either compound 85 or compound 86, depending on the position of the remaining double bond (Fig. 47). The distinction between 85 and 86 was made via hydrolysis to humulinic acid, which proved that the double bond in the 4-methyl-3-pentenoyl side chain had been hydrogenated preferentially. Thus trans dihydro-isohumulone, obtained as described above, has been identified as 2-(3-methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(4-methylpentanoyl)-2-cyclopentenone (85).

6.2. TETRAHYDRO-ISOHUMULONES.

Hydrogenation of the mixture of cis and trans isohumulones yields tetrahydro-isohumulones, which could not be characterized unequivocally (1,2). Two pathways are possible for the preparation of cis and trans 2-(3-methylbutanoyl)-5-(3-methylbutyl)-3,4-dihydroxy-4-(4-methylpentanoyl)-2-cyclopentenones or the tetrahydro-isohumulones (87 and 88 respectively, Fig. 47). The method via isomerization of tetrahydrohumulone should necessarily be followed by separation of the tetrahydro-isohumulones. On the other hand, hydrogenation of the individual isohumulones using palladium on carbon as catalyst affords the respective isomeric tetrahydro-derivatives (molecular formula $C_{21}H_{34}O_5$) (3,4). The spectrometric data resemble very much those of the corresponding isohumulones, except for the absence of the ^1H-NMR signals of the sp^2-methine protons and of the methyl groups on the double bonds. All absorptions occur in a small spectral region between δ 1 and δ 3.



85 86 87 88 89 90 91 92

Fig. 47. Structural formulae of the two dihydro-isohumulones (85,86), cis and trans tetrahydro-isohumulones (87,88), cis and trans tetrahydro-isocohumulones (90,91), 4-deoxytetrahydrohumulone (89) and 4-deoxytetrahydrocohumulone (92).

An independent structural proof for the tetrahydro-isohumulones was provided by the synthesis from phloroglucinol via 4-deoxytetrahydrohumulone. The same reaction sequence has been pursued as described for the synthesis of 4-deoxyhumulone (see 2.4.1.). The tetrahydro-isohumulones have been isolated by chromatography (4).

The cis and trans tetrahydro-isocohumulones (5) or 5-(3-methylbutyl)-3,4-dihydroxy-4-(4-methylpentanoyl)-2-(2-methylpropanoyl)-2-cyclopentenones (90 and 91, respectively, Fig. 47) were obtained by hydrogenolysis of colupulone (see

12.3.) to 4-deoxytetrahydrocohumulone (92, Fig. 47), oxidation and isomerization.

When tetrahydro-isohumulones are added to beer, the resulting solution is turbid and it foams excessively (6). These disadvantages can be avoided by brewing beer with tetrahydro-alpha acids, which behave identically as the alpha acids (3). They are more bitter than the iso-alpha acids and the bacteriostatic activity is more pronounced. Less is needed to reach a given bitterness level than with the iso-alpha acids, but the main advantage is the light stability, thus preventing the formation of off-flavours (7) (see also 6.5.1.). Since the tetrahydro-iso-alpha acids can be obtained by hydrogenolysis of the hop beta acids and subsequent air oxidation (see 12.3.), this procedure presents a possible method for utilizing the non-bitter hop beta acids as potential source for bitter contributions to beer (3). This has been realized on laboratory scale, but it failed in large-scale practice. The tetrahydro-isohumulones are much less soluble than the corresponding isohumulones and this can lead to gushing problems.

Preparation of the tetrahydro-isohumulones.

Cis or trans isohumulone (1 g; 2.76 x 10^{-3} mol) is hydrogenated in methanol (50 ml) using palladium on carbon (5%; 0.250 g). After the uptake of 2 mols hydrogen the catalyst is filtered off and the residue, after evaporation of the solvent, is recrystallized from iso-octane.

Cis tetrahydro-isohumulone (87).

Melting point : 67.5-69°C; $[\alpha]_D$ = +21.6.

UV : λmax (ε) : 273 (13730) nm (shoulder) and 254 (17690) nm in EtOH, 269 (14640) nm (shoulder) and 249 (18300) nm in EtOH : NaOH 0.1 N

IR : νmax : 3450, 2960, 1698, 1625, 1580, 1472, 1390, 1372, 1328, 1275, 1234, 1168 and 1135 cm^{-1}.

^1H NMR (220 MHz; $CDCl_3$; TMS) : δ : 0.87 (6H, d, J = 6.5 Hz); 0.97 (6H, d); 1.19 (2H, m); 1.47 (4H, m); 1.70 (1H, m); 2.17 (1H, m); 2.54 (2H, t, J = 6.0 Hz); 2.74 (2H, d, J = 7.0 Hz); 3.05 (1H, t, J = 7.0 Hz).

Trans tetrahydro-isohumulone (88).

Melting point : 75.5-78°C; $[\alpha]_D$ = -1.5.

UV : λmax (ε) : 272 (11300) nm (shoulder) and 254 (14600) nm in ethanol; 269 (7300)

nm and 230 (7300) nm in EtOH : HCl 0.1 N; 270 (1300) nm (shoulder) and 251 (16600) nm in EtOH : NaOH 0.1 N.

IR : vmax : 3500, 2960, 1698, 1632, 1583, 1472, 1390, 1374, 1280, 1158, 1133 and 1074 cm^{-1}.

^1H NMR (220 MHz; $CDCl_3$; TMS) : δ : 0.86 (6H, d); 0.98 (6H, d); 1.16 (2H, m); 1.42 (5H, m); 1.93 (1H, m); 2.14 (1H, m); 2.50 (2H, m); 2.70 (2H, d, J = 7.0 Hz); 2.85 (1H, m).

6.3. NEOHYDRO-ISOHUMULONES AND NEOHYDRO-ISOCOHUMULONES.

Prolonged hydrogenation of the tetrahydro-isohumulones using palladium on carbon leads to the cis and trans neohydro-isohumulones or 2,5-bis(3-methylbutyl)-3,4-dihydroxy-4-(4-methylpentanoyl)-2-cyclopentenones (93 and 94, respectively; Fig. 48) (molecular formula $C_{21}H_{36}O_4$) (1). In an analogous way the cis and trans neohydro-isocohumulones or 5-(3-methylbutyl)-3,4-dihydroxy-4-(4-methylpentanoyl)-2-(2-methylpropyl)-2-cyclopentenones (molecular formula $C_{20}H_{34}O_4$) are obtained (95 and 96, respectively; Fig. 48) (5). In the ^1H NMR spectra the signals of the methyl groups and of the methine proton, derived from the 2-methylpropanoyl side chain in the tetrahydro-isohumulones, are found at higher field, which confirms the transformation to a 2-methylbutyl group. Thus, the β-tricarbonyl chromophore has been reduced to a β-dicarbonyl unit. The absorption maxima at 253 (ε 11600) nm in acidic methanol and 274 (ε 17200) nm in alkaline methanol indicate a substituted cyclopentane-1,3-dione structure rather than a 2-acylcycloalkanone, which has an absorption maximum at longer wavelenghts (8,9).

<u>Preperation of the neohydro-isocohumulones.</u>

Cis or trans tetrahydro-isocohumulone (0.2 g; 5.68 x 10^{-4} mol) in methanol (4 ml) is hydrogenated using palladium on carbon (0.050 g). After uptake of 2 mols hydrogen, the catalyst is filtered off and the residue, after evaporation of the solvent, is purified by chromatography on silica gel. Elution with hexane : ethyl acetate 7:3 affords cis neohydro-isocohumulone (0.134 g; 70%). For the isolation of trans neohydro-isocohumulone (0.134 g; 70%) the eluent is used in a ratio of 21:4. This compound can be recrystallized from hexane yielding white needles (melting point : 128-129°C).

<u>Cis neohydro-isocohumulone (95).</u>

UV : λmax (ε) : 254 (15120) nm in MeOH : HCl 0.1 N; 271 (18700) nm in MeOH : NaOH 0.1 N.

IR : vmax : 3420, 1700, 1640, 1380, 1340, 1135 cm^{-1}.

^1H NMR (60 MHz; $CDCl_3$; TMS) : δ : 0.88 (18H, m); 1.5 (9H, m); 2.12 (2H, m); 2.5 (3H, m).

<u>Trans neohydro-isocohumulone (96).</u>

UV : λmax (ε) : 250 (11750) nm in MeOH : HCl 0.1 N; 273 (21300) nm in MeOH : NaOH 0.1 N.

IR : vmax : 3420, 1700, 1650, 1375, 1330, 1130, 1080 cm^{-1}.

^1H NMR (60 MHz; $CDCl_3$; TMS) : δ : 0.85 (18H, m); 1.4 (9H, m); 2.12 (2H, m); 2.4 (2H, t, J = 7.0 Hz); 2.75 (1H, m).

6.4. COMPOUND 97 or 2-(3-methylbutyl)-3-hydroxy-4-(4-methylpentanoyl)-5-(2-methylpropanoyl)cyclopentanone.

A compound (molecular formula $C_{20}H_{34}O_4$) has been isolated (20%) from the hydrogenation reaction mixture of tetrahydro-isocohumulone in methanol using palladium on carbon as catalyst. In the ^1H NMR spectrum the presence of a 2-methylpropanoyl side chain is observed, while a singlet at δ 2.8 is attributed to a ring methylene group. These data indicate structure 97 (Fig. 48) rather than the alternative structure 98 (Fig. 48). The UV absorption maxima at 284 (ε 5690) nm in acidic methanol and 312 (ε 14000) nm in alkaline methanol provide further proof for the acylcyclopentanone structure (9-11).

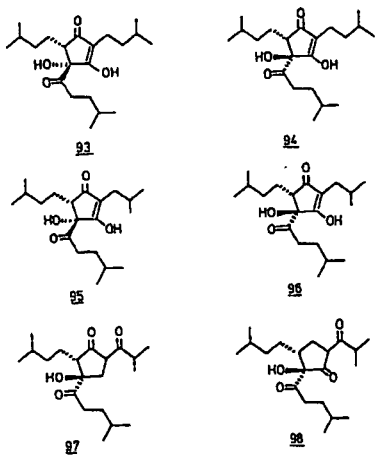


Fig. 48. Structural formulae of the neohydro-isohumulones (93,94), the neohydro-isocohumulones (95,96) and the reduced compounds (97,98).

6.5. THE RHO-ISOHUMULONES.

6.5.1. SUNSTRUCK FLAVOUR OF BEER.

Upon exposure to sunlight, beer develops an offending odour and taste, known as sunstruck flavour. Kuroiwa attributed this off-flavour to the formation of 3-methyl-2-butene-1-thiol via photolysis of the iso-alpha acids in the presence of sulfur-containing amino acids or proteins (12,13). This compound has been synthesized and compared to the sunstruck flavour (6). Also, the thiol has been identified unequivocally by irradiation of beer, concentration of the solution and gas chromatographic determination via flame photometric detection (14). The maximum concentration is 10^{-3} mg.l^{-1}. Even in these minute quantities the effect on beer quality is disastrous. Similar results have been obtained starting from model mixtures that simulate the beer medium. For this purpose ascorbic acid (0.2 g), isohumulone (0.035

g), L-cysteine (0.080 g) and riboflavin (0.040 g) are added to a buffer solution with pH 4.2 (water : ethanol 97.75:2.25) and the resulting solution is saturated with carbon dioxide.

The 3-methyl-2-butene-1-thiol is formed by recombination of a 3-methyl-2-butenyl radical and a thiol radical, possibly derived from sulfur-containing proteins present in beer. The alkenyl radical originates from photolysis of the acyloin group, which is composed of the tertiary alcohol function at C-4 and the carbonyl group of the 4-methyl-3-pentenoyl side chain at C-4 of the iso-alpha acids (Fig. 49). Alternatively, direct α-cleavage leading to the 3-methyl-2-butenyl radical may be envisaged. Since the iso-alpha acids do not absorb visible light, a sensitized process must occur via the mediation of riboflavin (vitamin B2) or polyphenols. The reaction is more efficient in reducing medium (ascorbic acid) and is suppressed by oxygen (15). The formation of the sunstruck flavour must occur in high quantum yield, although in-vitro experiments have not yet demonstrated this assumption. The intermediate 4-methyl-3-pentenoyl radical decarbonylates probably very rapidly since coupling products with thiol or sulfide radicals have not been detected. Specific changes in the 4-methyl-3-pentenoyl side chain of the iso-alpha acids may stabilize beer against the influences of light. Thus, the sunstruck flavour has not been observed when the double bond or the carbonyl group of this particular side chain are reduced, i.e. with the tetrahydro-isohumulones or with the so-called rho-isohumulones (p-isohumulones). In the rho-isohumulones the photolysis is blocked, while for the tetrahydro-isohumulones no stabilized 3-methyl-2-butenyl radical can be formed (16). As an application some beers are brewed with pre-isomerized extract that has been reduced with sodium borohydride (see 6.5.2.) (17). These beers can be stored in clear uncoloured glass bottles. In contrast, beers, that are bittered with iso-alpha acids must be stored in dark-brown or dark-green bottles. Otherwise, they are rapidly degraded through the development of the sunstruck flavour. Some beers, naturally low in riboflavin and in iso-alpha acids, are much less sensitive to sunstruck flavour than could be expected. We suggest that this could be related to brewing in copper vessels (copper destroying riboflavin?).

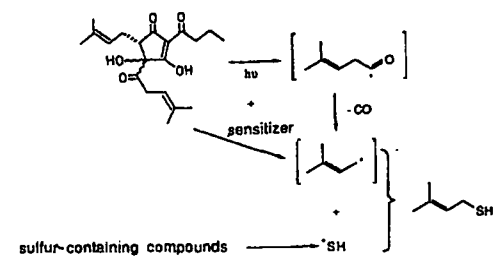


Fig. 49. Formation mechanism of 3-methyl-2-butene-1-thiol.

6.5.2. BOROHYDRIDE REDUCTION OF THE ISOHUMULONES.

Reaction of the isohumulones with sodium borohydride leads to reduction of the carbonyl group in the 4-methyl-3-pentenoyl side chain to a secondary alcohol function. Since a new chiral centre is introduced, four diastereo-isomeric reaction products are possible. They are the rho-isohumulones or 2-(3-methylbutanoyl)-5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(1-hydroxy-4-methyl-2-pentenyl)-2-cyclopentenones (18,19). Separation by counter-current distribution (CCD) of the individual stereo-isomers is only possible when the reduction is carried out on cis and trans isohumulone separately. In the two-phase system iso-octane : aqeous buffer pH 5.4, trans rho-1-isohumulone (99, Fig. 50) has a K value of 1.0 after 450 transfers; trans rho-2-isohumulone (100, Fig. 50) is distributed with a K value of 0.4. The ratio rho-1 : rho-2 is 3 : 1. For the separation of the cis rho-isohumulones 600 transfers are necessary, whereby the two epimers rho-1 (101, Fig. 50) and rho-2 (102, Fig. 50) have distribution coefficients of 0.39 and 0.25, respectively, in a ratio of 1:1.

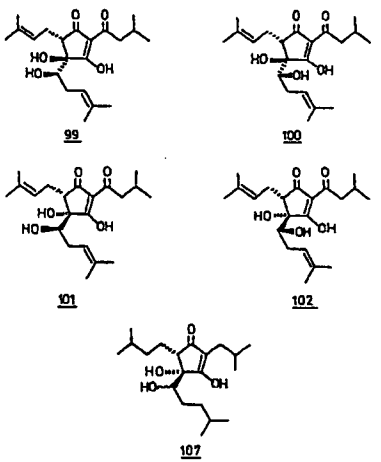


Fig. 50. Structural formulae of the rho-isohumulones (99-102) and of compound 107.

Trans rho-1-isohumulone (99, Fig. 50) is a crystalline compound with a melting point of 80°C and a specific optical rotation of -39.3 in methanol at the Na_D-line, while the rho-2-epimer is a light-yellow oil with a specific optical rotation of -11 in methanol. The molecular formula is $C_{21}H_{32}O_5$. The carbonyl absorption around 1720^{-1}, present in the IR spectrum of the isohumulones, has disappeared. The UV spectra are very similar. The changes in the ^1H NMR spectra, as compared to those of the isohumulones, are :

- the absorption for the protons of the methylene group in the 4-methyl-3-pentenoyl side chain at δ 3.3 is absent and new features are a doublet of doublets for 99 and a triplet for 100 at δ 3.1;
- the presence of an additional hydroxyl proton at δ 7.3 for 99 and at δ 7.7 for 100. The most sterically hindered structure is trans p1-isohumulone, which may be explained by

hydrogen bridge formation between the new alcohol function and the oxygen atom at C-3 in the ring. From the inspection of Dreiding models it appears that the torsional angle between the long vicinal side chains at C-4 and C-5 is small, leading to unequal population of rotamers. Thus the absolute configuration of the new chiral centre is S for **99** and R for **100**. Chemical proofs for the correctness of the structures of the rho-isohumulones include the absence of humulinic acid upon boiling in alkaline conditions and the isolation of dehydrohumulinic acid (see 8.4.4.2.), in addition to 4-methyl-3-pentenal, upon treatment with sodium periodate.

Cis rho-1-isohumulone (**101**, Fig. 50) is also a solid with melting point 78-80°C and a specific optical rotation at the Na_D-line of +38.3 in methanol. Cis rho-2-isohumulone is an oil with a specific optical rotation of +12.0 at the Na_D line in methanol. The spectral data and the chemical behaviour are almost identical to those of the trans isomers. Cis rho-1-isohumulone has the S-configuration at the new chiral centre, while cis rho-2-isohumulone has the R-configuration. In an analogous way all rho-1-iso-alpha acids may be obtained, among others the homologous rho-isocohumulones or 5-(3-methyl-2-butenyl)-3,4-dihydroxy-4-(1-hydroxy-4-methyl-2-pentenyl)-2-(2-methylpropanoyl)-2-cyclopentenones (**103-106**, Fig. 51) (5). The relative ratios of the rho-isohumulones **99:100:101:102** in the crude reaction mixture are 6:20:37:37. The bitterness of the rho-isohumulone is maybe slightly weaker than that of the isohumulones, while no significant differences are noted among the individual stereo-isomers.

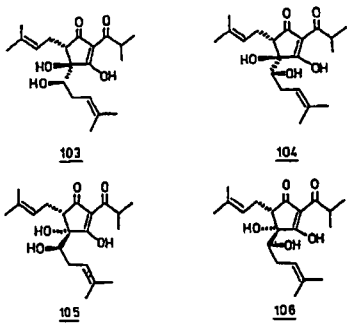


Fig. 51. Structural formulae of the rho-isocohumulones (**103-106**).

Some years ago, Pfizer Inc. marketed a product labelled Redi-Hop which was a sodium borohydride isomerized-reduced hop extract conditioned for post-fermentation addition. This commercial rho-iso-alpha acids product was discontinued. Recently, Pfizer entered the market again with more traditional forms of hop extracts and isomerized preparations which are characterized by a remarkably high content of either alpha acids or iso-alpha acids (20).

Preparation of the rho-isohumulones.

Cis or trans isohumulone (1.5 g; 4.14 x 10^{-3} mol) in iso-octane (50 ml) is added, under stirring, to sodium borohydride in water (50 ml) during 18 h at ambient temperature. For cis isohumulone four equivalents of sodium borohydride (0.175 g) are used, for trans isohumulone eight equivalents (0.35 g). The reaction mixture is subsequently acidified to pH 1 (HCl), extracted with iso-octane, dried and concentrated to a viscous oil (1.4 g), which is further separated by CCD in the two-phase system iso-octane : aqeous buffer pH 5.4. After 450 transfers the K values for trans rho-1-isohumulone and trans rho-2-isohumulone are 1.0 and 0.4, respectively. The K values for cis rho-1-isohumulone and cis rho-2-isohumulone are, after 600 transfers, 0.39 and 0.25, respectively.

6.6. COMPOUND **107** or cis 5-(3-methylbutyl)-3,4-dihydroxy-(1-hydroxy-4-methylpentyl)-2-cyclopentenone.

Reduction of cis tetrahydro-isocohumulone (**90**, Fig. 47) with sodium borohydride leading to the expected cis rho-tetrahydro-isocohumulones, also yields some cis 5-(3-methylbutyl)-3,4-dihydroxy-(1-hydroxy-4-methylpentyl)-2-cyclopentenone (**107**, Fig. 50) (5). This mixture of epimers is also obtained by the same reduction procedure carried out on cis neohydro-isocohumulone (**95**, Fig. 48) (5). The molecular formula is $C_{20}H_{36}O_4$. The UV spectra are similar to those of the neohydro-isocohumulones. The ^1H NMR spectrum proves that the acyl side chain is absent and the multiplet at δ 3.6 for one proton indicates that the 4-methylpentanoyl group has been reduced.

6.7. REFERENCES TO CHAPTER 6.

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CHAPTER 7
OXIDIZED DERIVATIVES OF THE ISOHUMULONES

7.1. NON-VOLATILE OXIDIZED DERIVATIVES.

The oxidized derivatives of the isohumulones can be obtained directly from humulone. They are discussed in the context of the oxidized derivatives of humulone (Chapter 4). The main compound, humulinone (24, Fig. 15), can not be prepared starting from the isohumulones, while the abeo-isohumulones (58-64, Figs. 24-26) are accessible from the isohumulones (see 4.10.)

7.2. VOLATILE OXIDIZED DERIVATIVES.

7.2.1. BEER OFF- FLAVOURS.

As stated before, beer has to keep for very long periods in these modern times of global commercialization. The slow generation of off-flavours, in this context, is of great importance. Sunstruck flavour is connected with iso-alpha acids, as previously discussed, but the other well known beer off-flavours mentioned in this Chapter, are not. However, a brief discussion is required, because research exploring possible connections with hop bitter acids has been carried out.

The stale and cardboard flavour of beer are caused by alkenals and alkadienals containing 6 to 10 carbon atoms, such as trans 2-nonenal (1-4). The diacetyl or 'buttery' off-flavour is caused by vicinal diketones, such as butane-2,3-dione (diacetyl) and pentane-2,3-dione (5-9) and by furan derivatives, such as furfural (see also 4.10.). The formation of unsaturated aldehydes is connected with the oxidative degradation of the isohumulones (10-15). In this process volatile aldehydes are generated, which undergo aldol-type condensations with other aldehydes, formed by oxidation of fats and higher alcohols (15). The vicinal diketones are formed by oxidative decarboxylation of 2-acetohydroxycarboxylic acids (5,6,9), such as 2-acetolactic acid (precursor of valine) and 2-acetohydroxybutyric acid (precursor of isoleucine) or from pyruvic acid via coupling of acetylcoenzyme A with acetaldehyde-thiamine pyrophosphate complex (6). The taste threshold values for these compounds are very low : less than 10^{-2} mg.l^{-1} (2,6) down to even 5×10^{-4} mg.l^{-1} for trans 2-nonenal, which is he lowest limit for an off-flavour beer component (3). Beer which contains these components in higher concentration, has a very unpleasant resinous taste. When the concentration is in the range of mg.l^{-1}, the beer is no longer drinkable. Some suppression of the off-flavour occurs upon addition of anti-oxidants, such as ascorbate